REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	25 Oct 95	

4. TITLE AND SUBTITLE Investigation into the Role of Oxidative Stress in the Mechanism of Dieldrin Hepatotoxicity in the B6C3F1 Mouse

5. FUNDING NUMBERS

6. AUTHOR(S)

Stephen Brichowski

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

AFIT Students Attending:

Indiana University

8. PERFORMING ORGANIZATION REPORT NUMBER

95-020D

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

DEPARTMENT OF THE AIR FORCE
AFIT/CI
2950 P STREET, BLDG 125
WRIGHT-PATTERSON AFB OH 45433-7765

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release IAW AFR 190-1
Distribution Unlimited
BRIAN D. Gauthier, MSgt, USAF
Chief Administration

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

19951031 114

14. SUBJECT TERMS	15. NUMBER OF PAGES 238
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT

Investigation into the Role of Oxidative Stress in the Mechanism of Dieldrin Hepatotoxicity in the B6C3F1 Mouse

Stephen Bachowski

Submitted to the faculty of the Indiana University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy in Toxicology
in the Department of Pharmacology and Toxicology,
Indiana University

September 1995

Accepted by the Graduate Faculty, Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Toxicology.

James E. Klaunig, Ph.D.

Doctoral Committee

Michael R. Vasko, Ph.D.

Victor Elharrar, Ph. D.

Mark A. Carfagna, Ph. D.

August 1, 1995

DEDICATION

To my wife Rachele for her love and encouragement

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. James Klaunig. His support and guidance were instrumental. His enthusiasm for research and his ability to critically review experimental design were paramount for the successful completion of this endeavor. In addition, I wish to express my appreciation to the other members of my committee, Dr. Michael Vasko, Dr. Victor Elharrar and Dr. Mark Carfagna, for their support and guidance. The diverse education I have received at Indiana University in the Pharmacology and Toxicology Department will be extremely valuable to me in the future.

I also want to acknowledge three assistant scientists: Yong Xu, Ghulam Kalimi and Ann Kwiatkowski. They provided good role models in the toxicology laboratories. Their strong work ethic, problem solving abilities and grasp of the literature encompass critical traits that lead to successful careers in science. I sincerely thank Yong Xu for his guidance and training in high pressure liquid chromoagraphy.

In addition, I want to thank my fellow students and colleagues. Thomas Baker, from whom I learned much of my *in vitro* skills; Kyle Kolaja, for the many scientific discussions we have had over the years on dieldrin and cancer; and Cythia Ketcham, Craig Zwickl, Rebecca Little and Jason Isenburg for their camaraderie over the years.

I thank Dr. Don Stevenson of Shell Oil Company and Dr. Earl Walborg of Dermigen, Inc. for their support in funding this research. I enjoyed the technical discussions we have had during this project. A special thanks to Dr. Stevenson for his expertise and historic perspective on dieldrin.

Finally, I wish to express my appreciation to the United States Air Force for selecting me for this program. I have always been proud to be a member of the Armed Forces of the United States of America. I look forward to applying the skills I have learned at Indiana University in the service to my country.

ABSTRACT

The production of reactive oxygen species (ROS) by toxic chemicals has been implicated in acute and chronic disease states, including cancer. This increase in cellular ROS can lead to a state of oxidative stress. Oxidative stress is a condition in which the pro-oxidant level within the cell exceeds the cell's capability to remove ROS or repair the damage they cause. Many organochlorinated compounds selectively induce hepatic tumors in mice but not rats. The mechanism for the induction of hepatic cancer by these compounds and the observed species selectivity of this effect are not known. It has been hypothesized that induction of oxidative stress may contribute to the observed hepatic toxicity and carcinogenicity of these compounds in mice. Dieldrin is one such compound and was used in this study to characterize the relationship between oxidative stress and the observed selective hepatotoxicity of dieldrin in mice. Mouse microsomes and hepatocytes showed an increase in the production ROS. This was not observed for the rat. Furthermore, dieldrin induced oxidative stress in mouse but not rat hepatocytes as evidenced by an increase in lipid peroxidation in mouse hepatocytes. *In vivo* experiments showed dieldrin to produce a dose-dependent and species-specific (mouse only) increase in hepatic malondialdehyde (MDA). Increases in urinary MDA and 8-hydroxy-2'-deoxyguanosine (oh8dG) were also observed in dieldrin treated mice. These increases coincided with the observed increase in DNA S-phase synthesis observed in mice. While dieldrin decreased hepatic Vit E in both mice and rats, no oxidative damage was observed in rats. Dieldrin induced oxidative stress in B6C3F1 mice was shown to be modulated by dietary Vit E. Hepatic MDA observed with dieldrin treatment in mice also decreased proportionally

with the increase of dietary Vit E. These studies suggest that the induction of oxidative stress may be a mechanism by which dieldrin and mouse specific chlorinated compounds selectively induce their toxic effects in mouse liver.

James E. Klaunig, Ph.D.

Victor Elharrar, Ph. D.

Michael R. Vasko, Ph.D.

Mark A. Carfagna, Ph. D.

August 1, 1995

SUMMARY

Oxidative stress has been implicated in acute and chronic disease states, including cancer. Many organochlorinated compounds are selective hepatocarcinogens in mice but not rats. Dieldrin is one such compound which selectively induces hepatic tumors in mice but not rats. The mechanism for the induction of hepatic cancer by dieldrin and the observed species selectivity of this effect is not known. It has been hypothesized that induction of oxidative stress by dieldrin may contribute to its observed hepatic toxicity and carcinogenicity in mice. To define this model, a species comparison was performed to evaluate the basal concentrations of antioxidants and oxidative damage in the livers of these rodents. This was contrasted with the susceptibility of these species and mouse strains to carcinogenesis. Basal concentrations of 8-hydroxy-2'-deoxyguanosine (oh8dG), glutathione (GSH), ascorbic acid (Vit C), uric acid (UA) and vitamin E (Vit E) were analyzed in the liver of 6-8 wk old F344 rats and C3H/He, B6C3F1 and C57Bl/6 mice by high pressure liquid chromatography (HPLC) detection methods. Next, this species and strain comparison was characterized by assaying the basal concentrations of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and glutathione peroxidase (GSH-Px). The results of basal oh8dG concentrations were consistent with the animal susceptibility to liver carcinogenesis.

Significantly higher concentrations were observed in mouse liver than in rat. In contrast hepatic Vit C and Vit E were both higher in rats than in mice. It is interesting to note that hepatic basal GSH concentrations in F344 rats were markedly lower than in mice. Furthermore, F344 rat livers had significantly lower basal GST activity than mouse strains and lower basal SOD activity ($P < 0.05$). However, the basal GSH-Px activity was only slightly lower in rat liver than in B6C3F1 mouse liver while no differences in basal catalase activity were observed between the species. The basal hepatic oh8dG concentrations correlated well with species liver susceptibility to carcinogenesis and the concentrations of the non-enzymatic antioxidants may be the more important parameter in regulating this level of oxidative damage between the species. B6C3F1 mice appear to have a higher basal level of oxidative stress compared to F344 rats; therefore, they may be more sensitive to the effects of ROS generated by xenobiotics than rats.

An *in vitro* system was used to characterize the generation of ROS and their effect on hepatocytes. Mouse and rat microsomes and primary hepatocyte cell cultures were used to demonstrate the production of ROS by dieldrin using an aromatic hydroxylation assay. Likewise, primary culture hepatocytes were used to determine the induction of oxidative stress as measured by malondialdehyde (MDA) and oh8dG. Mouse microsomes and hepatocytes showed an increase in ROS as measured by the increase in hydroxyl adducts of salicylic acid. This was not observed in rat microsomes

or hepatocytes. Dieldrin induced oxidative stress in mouse but not rat hepatocytes as evidenced by a dose-dependent increase in lipid peroxidation in mouse hepatocytes. Curiously, cellular Vit E deceased in both rat and mouse hepatocytes treated with dieldrin while cellular Vit C increased. This decrease in cellular Vit E was accompanied by an increase in lipid peroxidation in mouse but not rat hepatocytes. Likewise, an increase in cellular oh8dG was only observed in mouse hepatocytes treated with dieldrin and not rat. This increase in oxidative stress, in mouse but not rat hepatocytes, with the depletion of cellular Vit E may not be that surprising since the rat does not appear to produce free radicals through the cytochrome P-450 system with dieldrin treatment. Thus, while the rat's antioxidant level was lowered, its pro-oxidant level had not risen to a degree to cause oxidative stress. Mouse hepatocytes, therefore, exhibited a greater sensitivity to the *in vitro* effects of dieldrin than rat hepatocytes. This species differences may be related to selective dieldrin-induced oxidative stress in mouse hepatocytes.

Having demonstrated a selective induction of oxidative stress in mouse hepatocytes, we next sought to correlate this effect with the selective induction of DNA synthesis observed *in vivo*. Many xenobiotics that promote tumor growth (tumor promoters) also cause an increase in hepatic DNA synthesis *in vivo*. This induction of DNA synthesis has been observed in previous studies with dieldrin selectively in mouse but not rat livers. Animals were given either 0, 0.1, 1.0 or 10 mg dieldrin/kg in their diet for 7,

14, 28 and 90 days. Non enzymatic antioxidants (Vit E, Vit C, GSH, UA) and oxidative stress markers (MDA, oh8dG) were again measured. These parameters were then compared to the induction of DNA S-phase synthesis observed in mice but not rats on dieldrin diet. Dieldrin produced a dose-responsive and species specific (mouse only) increase in hepatic MDA; however, hepatic oh8dG did not change. Increases in urinary MDA and urinary oh8dG were observed in dieldrin treated mice. These increases coincided with the observed increase in DNA S-phase synthesis observed in mice. At early time points, hepatic MDA and urinary oh8dG were high in mice as was the DNA S-phase synthesis. However, by 90 days, both hepatic MDA and urinary oh8dG were either the same or below control in mice; likewise, DNA S-phase synthesis was also lower. This may be due to improved elimination of MDA from the liver or a decrease in hepatic oxidative stress resulting from higher Vit C concentrations. Subsequent evaluation showed that dieldrin-induced oxidative stress correlated with a decrease in hepatic Vit E in mice at early time points (r^2=0.425, P<0.001 at 7 days and r^2=0.302, P<0.001 at 14 days). Dieldrin also decreased hepatic Vit E in rats; however, rats having higher basal concentrations of Vit E and Vit C were still able to prevent hepatic MDA formation. A linear regression analysis showed a good correlation between MDA, Vit C, and Vit E when compared to DNA synthesis in the subchronically dieldrin treated mice. These studies suggest that the induction of oxidative stress may be a

mechanism by which dieldrin and other chlorinated compounds produce their selective hepatic toxic effects (including cancer) in mice.

If oxidative stress has a critical role in the selective action of dieldrin, the effect of dieldrin in mice should be modulated by antioxidants. To test this hypothesis, an *in vivo* study was performed using 0, 50, 250 and 450 mg Vit E/kg diet with and without 10 mg dieldrin/kg. The serum and hepatic Vit E from dieldrin treated animals correlated with dietary intake (serum Vit E at 14 and 28 days: r^2=0.60; r^2=0.688 and hepatic Vit E at 14 and 28 days: r^2= 0.961; r^2= 0.942). Hepatic Vit E was reduced about 35% by the addition of 10 mg dieldrin/kg diet. Furthermore, the hepatic MDA observed with dieldrin treatment decreased proportionally with the increase of dietary Vit E.

In summary, dieldrin generates ROS in mouse but not rat hepatocytes. This generation of free radicals results in oxidative stress in both isolated mouse hepatocytes and whole liver. Even though dieldrin lowers hepatic Vit E in both mice and rats, this effect does not result in oxidative stress in the rat liver. The induction of oxidative stress *in vivo* correlates with the induced DNA S-phase synthesis suggesting that dieldrin's selective hepatocarcinogenic action in the mouse may be related to the induction of oxidative stress.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

[4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid	Wy-14,643
2'-deoxyguanosine	dGuo
2,3-dihydroxybenzoic acid	2,3-DHBA
2,5-dihydroxybenzoic acid	2,5-DHBA
8-hydroxy-2'-deoxyguanosine	oh8dG
Activating Protein 1	AP-1
Aldrin-derived dicarboxylic acid	ADA
B cell leukemia/lymphoma 2	bcl-2
Body Weight	BW
Catalase	CAT
Cytochrome P-450	Cyt P-450
Deoxyribonucleic Acid	DNA
di(2-ethylhexyl)phthalate	DEHP
Dichlorodiphenyltrichloroethane	DDT
Gap Junctional Intercellular Communication	GJIC
Glutathione	GSH
Glutathione Peroxidase	GSH Px
Glutathione S-transferase	GST
Hexachlorocyclohexane	HCH
High Pressure Liquid Chromatography	HPLC

Inhibitory κB	IκB
Lactate Dehydrogenase	LDH
Lipid Peroxidation	LPO
Liver Weight	LW
Liver Weight/Body Weight	LW/BW
Malondialdehyde	MDA
Messenger RNA	mRNA
Mitochondrial DNA	mtDNA
Myeloperoxidase	MPO
National Cancer Institute	NCI
National Toxicology Program	NTP
Nitric Oxide	NO
Nuclear Factor κB	NF-κB
Pearson's Coefficient	r^2
Pentachloroketone	PCK
Phosphate Buffer Saline	PBS
Polychlorinated Biphenyls	PCB
Polyunsaturated Fatty Acids	PUFA
Reactive Oxygen Species	ROS
Ribonucleic Acid	RNA
Salicylic Acid	SA
Smooth Endoplasmic Reticulum	SER

Superoxide Dismutase	SOD
Tetrachlorodibenzo-p-dioxin	TCDD
Tetradecanoylphorbol-13-Acetate	TPA
Tumor Necrosis Factor alpha	TNFα
Uric Acid	UA
Vitamin C (Ascorbic Acid)	Vit C
Vitamin E (α-tocopherol)	Vit E

PURPOSE AND OVERVIEW

An increase in reactive oxygen species (ROS) is a pervasive phenomenon resulting from the *in vitro* or *in vivo* treatment of hepatocytes with tumor promoting xenobiotics. When xenobiotics create ROS, they can add to the level of oxidants endogenously produced by the cell and override the antioxidant defense system (Figure 1). These xenobiotics may act through a variety of different mechanisms to induce oxidative stress (i.e., peroxisome proliferation, direct production of free radicals by cytochrome P-450 system, mitochondria dysfunction or by altering the antioxidant defense system of the cell). The increased oxidative stress can cause lipid peroxidation resulting in altered cellular homeostasis (i.e., increased calcium, pH modulation or altered gap junction intercellular communication); activation of oncogenes such as Activating Protein-1 (AP-1), Nuclear Factor κB (NF-κB) or B cell leukemia/lymphoma 2 (bcl-2); or damage to DNA.

The formation of ROS has been proposed as a mechanism for chemically induced carcinogenesis by several investigators (Cerutti, 1991; Cerutti and Trump, 1991; Kensler and Trush, 1984; Borek 1991). However, the exact pathways mediated by this process still remain to be identified. DNA adduct formation by free radicals or hydroxylation of DNA has been suggested for the initiation step in carcinogenesis but the promoting ability of many xenobiotics still remains unclear. In 1971, Hadler first suggested that mitochondrial mutations lead to a modified growth advantage for

initiated cells. Later, Solt and Farber (1976) introduced a broader definition in which promotion occurs when initiated hepatocytes are resistant to the effects of xenobiotics thus giving them a selective growth advantage. Still, other investigators have examined the role of cell proliferation or inhibition of apoptosis in initiated cells as a mechanism of carcinogenesis.

Many chlorinated compounds selectively induce hepatic cancer in mice but not rats. Dieldrin is a typical example of one such compound. Initial studies have shown dieldrin to induce centrilobular DNA synthesis in B6C3F1 mice but not F344 rats (Stevenson et al., 1995). This phenomena was partially reduced by the addition of 200 ppm Vit E to the diet. This research initiative focused on identifying the selective action of dieldrin in B6C3F1 mice. In brief, dieldrin's role in ROS generation was examined. Microsomes and whole cells were monitored for the generation of ROS. Also, primary hepatocytes were studied to see if the ROS generated resulted in damage to the cell. This damage was assessed by the level of MDA and oh8dG generated by dieldrin. Next, *in vivo* studies were performed to determine if dieldrin changes antioxidant levels and if these changes correlate to the observed increase in DNA synthesis. Finally, modulation of dieldrin's cellular effects by dietary Vit E were examined *in vivo* to determine if dieldrin's action was reversable.

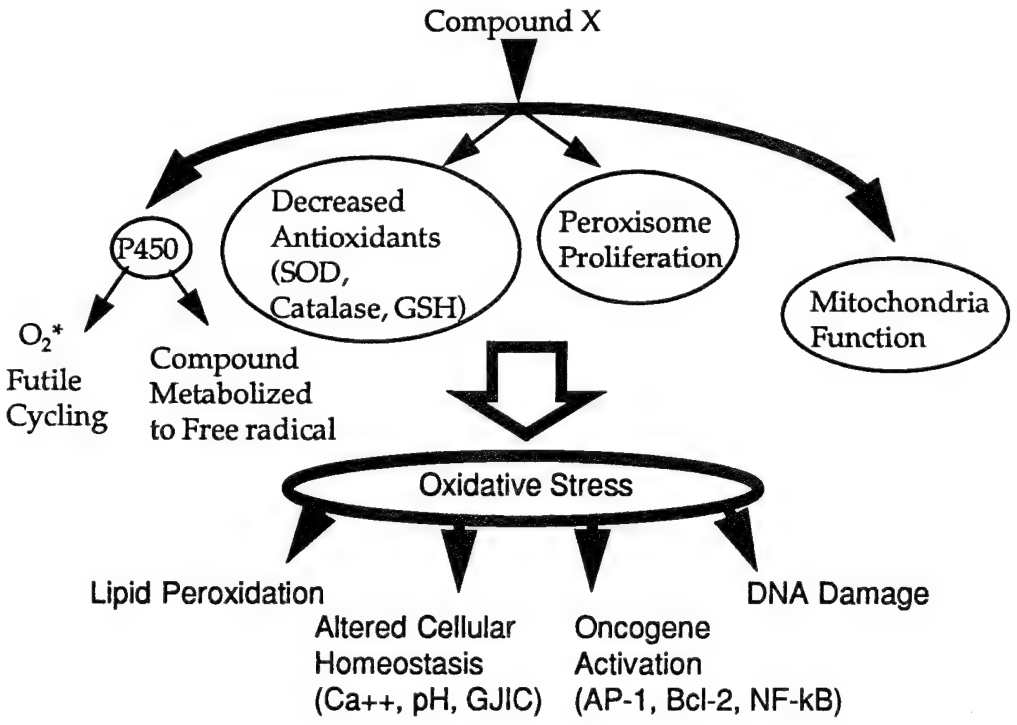


Figure 1: Xenobiotically Induced Cellular Oxidative Stress

When a xenobiotic enters a cell, it may change the antioxidant/pro-oxidant balance within the cell in favor of the pro-oxidants. This results in a state of oxidative stress. An increase in oxidative stress can result from: generation of superoxide by futile cycling in the cytochrome P-450 system, generation of hydrogen peroxide by peroxisomes, mitochondria dysfunction or a decrease in the basal levels of cellular antioxidants. The resulting oxidative stress may result in cell death, cellular damage (lipid peroxidation or oxidized DNA nucleotides) or activation of oncogenes.

HYPOTHESIS AND AIMS

HYPOTHESIS: Dieldrin induces oxidative stress which contributes to its selective chronic hepatotoxic effects in mice.

Aim 1: Determine if differences exist among rodent species in the antioxidant/oxidant balance.

Animal models which simulate carcinogenesis in humans can be utilized to identify chemical carcinogens and chemopreventive agents that might be involved in human carcinogenesis. It is, therefore, important to have basic data on animal strain and species differences which impact on the neoplastic process and its mechanism. The rate of spontaneous hepatic tumor formation varies greatly among the three common strains of mice used in toxicological bioassays. C3H/HeJ mice show a 50 times higher relative susceptibility to spontaneous liver tumors than C57BL/6 mice. Likewise, B6C3F1 mice, derived from segregating crosses between C3H/HeJ and C57BL/6 mice, are intermediate in the sensitivity to the two parental strains (Drinkwater and Bennett, 1991). In contrast to the mouse, no major differences are observed in spontaneous hepatic tumor formation in rat strains. Therefore, the F344 rat, which is the standard used by the National Cancer Institute (NCI), was used (Diwan et al., 1991). In aim 1, basal levels

of oxidative stress and antioxidants in these rodents were measured to ascertain if a relationship existed with their reported rate of spontaneous tumor formation.

Aim 2: Determine if dieldrin perturbs the hepatic antioxidant/oxidant balance in rodent hepatocytes.

Part 1: Does dieldrin produce ROS in rodent microsomes?

Part 2: Does dieldrin produce ROS in rodent primary hepatocyte cell cultures?

Part 3: Does dieldrin produce oxidative stress in rodent hepatocyte primary cell cultures?

An increase in ROS may either be a direct result of metabolism of a xenobiotic or the xenobiotic perturbing the cellular homeostasis. To test the former possibility, an aromatic hydroxylation assay was used on microsomal fractions and hepatocytes utilizing the salicylate method of Grootveld and Halliwell (1986). To test the second possibility, levels of cellular non-enzymatic antioxidants (Vit E, Vit C, GSH and UA) and indicators of oxidative stress (MDA and oh8dG) were measured.

Dieldrin, an organochlorinated insecticide, is a selective hepatocarcinogen in mice. Chlorinated hydrocarbons have been shown to

induce lipid peroxidation. Hexachlorocyclohexane (HCH), Carbon tetrachloride, hexachlorobenzene and tetrachlorodibenzo-p-dioxin (TCDD) induced lipid peroxidation in female Sprague-Dawley rats (Goel et al., 1988). Dichlorodiphenyltrichloroethane (DDT) caused lipid peroxidation in male Wistar rats (Barros et al., 1994). Likewise, endrin and lindane produced lipid peroxidation and ROS in male rats (Hassen et al., 1991; Bagchi et al., 1993). The mechanism behind this oxidative damage has not been thoroughly investigated. Parke and Ioannides (1990) suggest futile cycling of compounds in the hepatocyte cytochrome P-450 system may be the cause. This futile cycling results in the generation of ROS. This production of ROS can then proceed to create oxidative stress in hepatocytes by overriding the antioxidant defense system.

Therefore, to examine these questions, microsomes and hepatocytes were examined for their ability to hydroxylate salicylic acid in response to dieldrin. Mouse and rat hepatocytes were then examined further for evidence of oxidative stress and changes in the non-enzymatic antioxidant defense system. The focus on non-enzymatic antioxidants was based on three points: 1) De and Darad (1991) examined antioxidants and aging in rats and only saw significant changes in non-enzymatic antioxidants, 2) if desired, diets can be supplemented with non-enzymatic antioxidants allowing for future dietary modulation studies to further examine this mechanism and 3) results from aim one suggested the non-enzymatic antioxidants were the

more important factors in the mouse and rat antioxidant/pro-oxidant balance. For the remaining studies, the B6C3F1 mouse and the F344 rat were utilized. Both of these rodents are standard test animals for bioassays by the NCI and the National Toxicology Program (NTP) (Cameron et al., 1985). The B6C3F1 mouse is a hybrid of the C57Bl/6 female and the C3H/He male. It was specifically developed for use in carcinogenesis screening studies by NCI. The Fisher 344 rat is an inbred rat with a low spontaneous tumor incidence. It was first developed by M.R. Curtis in 1920 and adopted as a standard rodent for general bioassay purposes by NCI in 1970 (Cameron et al., 1985).

Aim 3: Determine if dieldrin produces hepatic oxidative stress in subchronically treated rats and mice.

Organic and inorganic peroxides have been shown to be tumor promoters but not tumor initiators in mouse skin (Slaga et al., 1981 and Klein-Szanto et al., 1982). Normally, ROS produced by basal oxygen metabolism and/or by chemical or physical induction are kept in check by the antioxidant defense system (Trush and Kensler, 1991; Breimer, 1990; and Hochstein and Atallah, 1988). A state of oxidative stress occurs when the increased ROS formation overpowers the antioxidants. Therefore, by concurrently comparing parameters which reflect oxidative damage and

assaying the levels of antioxidants, the degree of oxidative stress in the liver can be ascertained.

The stimulation of DNA S-phase synthesis induced by dieldrin in the rodent liver was also measured. The stimulation of DNA synthesis in subchronic studies is a predictive tool to evaluate the carcinogenicity of xenobiotics (Busser and Lutz, 1987; Butterworth and Goldsworthy, 1992). Initial work with gap junction intercellular communication (GJIC) showed the antioxidant, Vit E, to reverse the inhibition of GJIC seen in mice. This *in vitro* inhibition of GJIC by carcinogens, similar to stimulation of DNA synthesis, is another predictive assay to characterize chemical carcinogens (Klaunig and Ruch, 1989). Therefore, in aim 3, a comparison was made on the stimulation of DNA S-phase synthesis and the antioxidant/pro oxidant balance in the rodent liver.

In aim 3, dieldrin was administered p.o. to B6C3F1 mice and F344 rats by being mixed with NIH-07 diet and formulated into pellets by a commercial supplier (Dyets, Inc. Bethlehem, PA). There were four dose groups, including control, consisting of 0, 0.1, 1 and 10 mg dieldrin/kg diet (based on Walker et al., 1972). 5 animals were used per dose group. Animals were sacrificed on days 7, 14, 28, and 90 based on *in vivo* DNA synthesis studies conducted by Stevenson et al., (1995). Livers were assayed for Vit E, Vit C, GSH, UA, MDA and oh8dG. Urine was assayed for MDA and oh8dG and serum was assayed for Vit E.

Aim 4: Determine if the oxidative stress induced by dieldrin in the B6C3F1 mouse can be modulated by dietary Vit E.

Initial work by Stevenson et al., (1995) has shown that Vit E can reverse the effect of dieldrin-induced DNA S-phase synthesis observed *in vivo*. In the previous section it was determined if dieldrin or ROS generated by dieldrin modulated the antioxidant defense system of mouse and rat livers, thus effecting the way this xenobiotic acts on these two species. In this experiment, dietary Vit E was varied in an attempt to modulate dieldrin's selective hepatotoxicity in mice. Specifically, dieldrin was administered p.o. to B6C3F1 mice and F344 rats by being mixed with NIH-07 diet and formulated into pellets by a commercial supplier (Dyets, Inc. Bethlehem, PA). Eight dose groups were used in this study: 0, 50, 250, 450 mg Vit E/kg diet (Kappius and Diplock, 1992) and 0, 50, 250, 450 mg Vit E/kg diet with 10 mg dieldrin/mg diet (based on Walker et al., 1972). Animals were sacrificed on days 14 and 28 based on *in vivo* DNA synthesis studies conducted by Stevenson et al., (1995). Livers were assayed for Vit E, Vit C, GSH, UA, MDA and oh8dG. Urine was assayed for MDA and serum was assayed for Vit E.

LITERATURE

A. REACTIVE OXYGEN SPECIES AND THEIR GENERATION

Oxidative stress has been defined as "a disturbance in the pro-oxidant/ antioxidant balance in favor of the former, leading to potential damage." (Sies, 1991). Mammalian cells generate reactive oxygen species (ROS) during normal metabolic processes. The cell has several ways to respond to ROS. It can either repair and remove the damaged nucleotides and lipid peroxidation by-products or directly reduce the ROS via enzymatic and non-enzymatic antioxidants.

ROS consist of reduced forms of diatomic oxygen: superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH\bullet$). Oxidizing agents, such as ROS, are substances which have a high affinity for electrons. When ROS capture electrons from other molecules, ROS are reduced (gain an electron). The other substances, in turn, are oxidized (lose an electron). Superoxide, the least reactive of these, cannot pass through cellular membranes due to its negative charge; but, it can be spontaneously or enzymatically dismutased to hydrogen peroxide (Radi et al., 1991). Hydrogen peroxide is electrically neutral and can pass through cellular membranes. It is typically present in cells at concentrations of 10^{-7} to 10^{-9} M. It's conversion to the more reactive hydroxyl radical occurs either by the

Haber-Weiss or the Fenton reactions (equations 1 and 2 in figure 2) (Freeman and Crapo, 1982; McCord, 1993). In the Haber-Weiss reaction, the ferric ion acts as a catalyst to convert the superoxide radical to the more reactive hydroxyl radical. The first step of this process involves the conversion of the ferric ion to its reduced form the ferrous ion. In the process, superoxide is converted to molecular oxygen (step 1 of equation 1). The second part of the Haber-Weiss reaction is in fact the classical Fenton reaction in which the ferrous ion reacts with hydrogen peroxide to form the hydroxyl radical, the hydroxyl ion and the ferric ion (Step 2 of equation 1). The hydroxyl radical, the most reactive of the ROS due to its unparied electron (half-life of 1 nanosecond), causes cellular damage in the immediate vicinity of its generation (Bankson et al., 1993).

Cells use oxygen in many physiological processes. Figure 3 illustrates some of the more prominent ROS producing processes resident in the cell and the antioxidants used to remove them. Peroxisomes, which break down fatty acids, generate hydrogen peroxide in the process. CAT located in the peroxisome can use this peroxide in detoxification reactions to metabolize compounds such as formaldehyde, alcohol and formic acid (de Duve et al., 1983; Tolbert et al., 1981). Likewise, the mitochondria, generates ROS during the cellular respiratory cycle and fatty acid catabolism. The breakdown of fatty acids generates acetyl coenzyme A which is used in the citric acid cycle (Mathews and van Holde, 1990). Both of these processes

involve the transfer of electrons to NADH and FADH for use by ubiquinone during cellular respiration. It has been estimated that 2 % of mitochondrial oxygen consumption goes toward the generation of ROS (Boveris et al., 1975). Mn-superoxide dismutase, as well as other antioxidants within the mitochondria, maintain the spurious ROS generated by this process at low levels. Work by Sohal and coworkers has in fact demonstrated a strong inverse correlation between production of ROS by mitochondria and longevity of mammalian species (Ku et al., 1993; Sohal et al., 1990). A third important site of free radical generation, the microsomal electron transport system (cytochrome P-450), requires electrons from NAD(P)H to produce partially reduced oxygen species. Normally, this process does not result in production of ROS but in the presence of selected xenobiotics, superoxide may be generated through futile cycling (Parke and Ioannides, 1990). Superoxide can then be converted to the more reactive hydroxyl radical via the Haber-Weiss reaction. Other enzymes within the cell directly produce ROS. These include: 1) xanthine oxidase which produces a molecule of hydrogen peroxide when catalyzing the conversion of hypoxanthine to xanthine and again in the conversion of xanthine to UA during purine degradation, 2) glucose oxidase which generates hydrogen peroxide in the presence of molecular oxygen (Mathews and van Holde, 1990), and 3) monoamine oxidases utilized in the breakdown of dopamine in neurons (Mathews and van Holde, 1990). The hydrogen peroxide generated can then form more reactive hydroxyl radicals

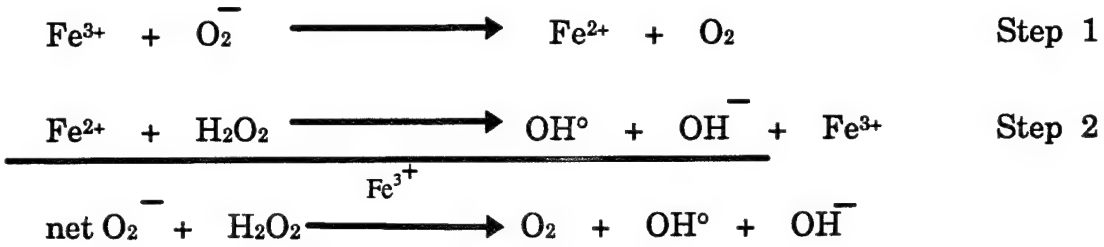
via the Fenton reaction (equation 2 in Figure 2) (Aust et al., 1985; Halliwell and Gutteridge, 1986). In addition, membrane associated enzymes utilized in the arachidonic acid cascade for prostaglandin, thromboxane and leukotriene synthesis produce peroxides. Cyclooxygenase converts arachidonic acid to prostaglandin endoperoxide while lipoxygenase catalyzes the conversion of arachidonic acid to hydroperoxyeicosatetraenoic acid (Freeman and Crapo, 1982; McCord, 1993).

In addition to cellular production of ROS, extracellular events can also mediate oxidative stress. Also, macrophages (Kupffer cells in the liver) generated ROS when activated to begin phagocytosis. Macrophages undergo oxidative burst which are catalyzed by a membrane bound enzyme NADPH oxidase (Equation 3) (Babior et al., 1973). An oxidative burst is characterized by the release of large amounts of superoxide from the macrophages. Macrophages can be activated by xenobiotics such as carbon tetrachloride and dieldrin (ElSisi et al., 1993; Hewett et al., 1988). The activated Kupffer cells can also increase production of tumor necrosis factor alpha (TNFα) which triggers the conversion of arginine to citrulline (equation 4) releasing nitric oxide (NO) via nitric oxide synthetase (Palmer et al., 1988). Furthermore, hypochlorate (HOCl) can be produced by myeloperoxidase (MPO) (Weiss et al., 1982; Freeman and Crapo, 1982). MPO is released from lysosomal granules of macrophages and other cells when they undergo oxidative burst. The NO radical, itself, can react with oxygen

to produce peroxynitrite, an intermediate capable of decomposing into the more active peroxide radical (Radi et al., 1991). Finally, UV light has been shown to generate free radical in the skin (Pathak et al., 1968).

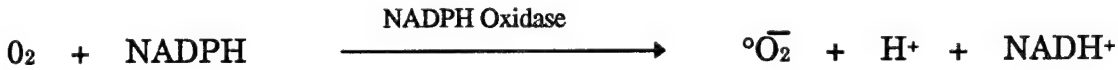
Figure 2: Reactions Involved in the Generation of ROS

Equation 1: The Haber-Weiss Reaction

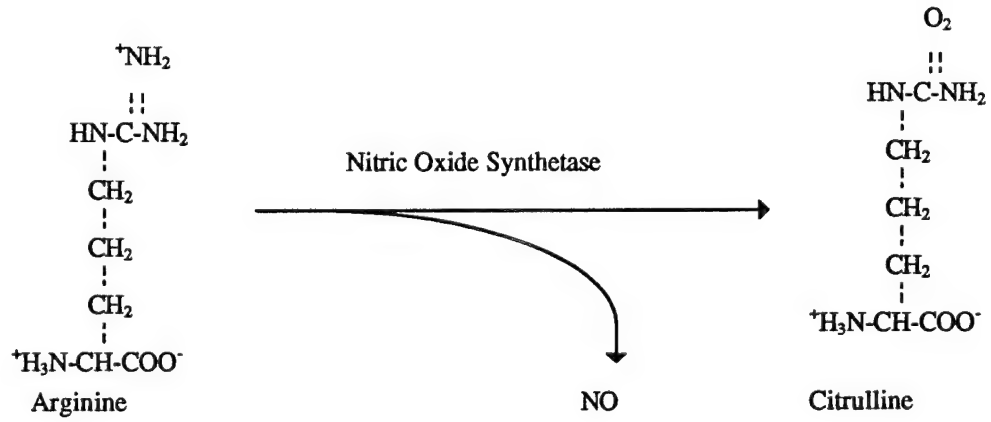


Equation 2: The Fenton Reaction

Equation 3: Formation of superoxide by NADPH Oxidase



Equation 4: Formation of Nitrogen Oxide



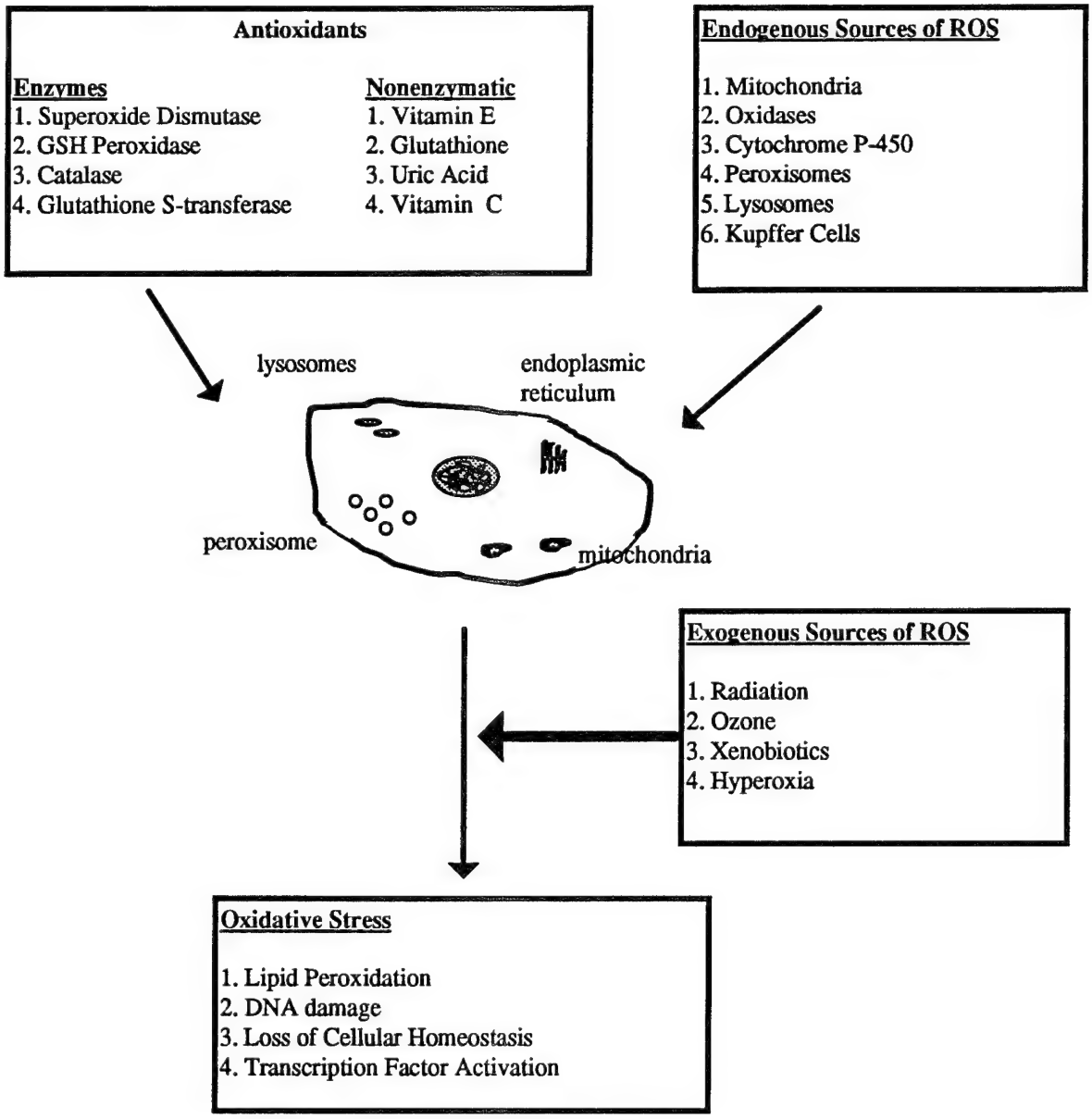


Figure 3: ROS Production and Disruption of Cellular Homeostasis

ROS can be created by a variety of endogenous sources. Normally, antioxidants within the cell remove these radical species. However, if exogenous factors increase the level of ROS, the pro-oxidant load exceeds the cells capabilities to remove these destructive species and a state of oxidative stress exists.

B. CELLULAR ANTIOXIDANT/OXIDANT SYSTEM

Hepatocytes live in a balance of free radical production, free radical scavengers and repair of damage caused by free radicals. The addition of xenobiotics to hepatocytes can upset this balance by: 1) directly forming free radicals, 2) inducing or altering enzymatic systems within the cell, such as cytochrome P-450, peroxisomes and mitochondria, which in turn generate free radicals, 3) depleting or inhibiting normal enzymatic and non-enzymatic antioxidant systems which scavenge the free radicals and protect the cell or 4) overburden the repair mechanisms within the cell.

The antioxidant defense system of the hepatocyte can be divided into two parts: non-enzymatic and enzymatic. The non-enzymatic antioxidant defenses consist of molecules such as UA, Vit E, Vit C and GSH that can directly act on free radicals. The enzymatic antioxidant defenses consist of SOD, CAT, GSH -Px and GST.

UA (Figure 4) has been shown to inhibit lipid peroxidation and protect erythrocytes against singlet oxygen damage (Ames et al., 1981; Smith et al., 1983). This may be due to UA's direct ability to act as an antioxidant or its ability to complex iron (Davies et al., 1986). Thus, lower levels of UA both lower the antioxidant level in tissue as well as make iron available for Haber-Weiss reactions. Parkinson's disease, which has been associated with oxidative stress in the substantia nigra, has been found to be associated with

lower levels of UA in this tissue (Church and Ward, 1993). Lower serum levels of UA have also been found in diabetics, cystic fibrosis and cancer patients (Pearl et al., 1993; Brown and Kelly, 1994; Lickl et al., 1989). Furthermore, work by Nyandieka and coworkers (1990) has shown high UA levels to reduce tumor incidence in rats induced by aflatoxin B1. Interestingly, man and other primates lack the enzyme, urate oxidase, which converts UA to allantoin in other species (Becker, 1993). Furthermore, 90% of UA in primates is reabsorbed in the kidneys and returned to the blood (Ames et al., 1981). Both of these facts suggest that UA is an important antioxidant in primates.

Vit E, Vit C and GSH can act both separately or in unison to reduce ROS. Vit E is a membrane bound antioxidant that can scavenge free radicals thereby preserving the integrity of the plasma membrane. The resulting tocopheroxyl radical formed from this process has higher stability and does not cause free radical damage (Sies and Murphy, 1991). The tocopheroxyl radical can go on to react with another lipid peroxide to form the tocopheryl quinone or be recycled by Vit C (Figure 5). Dihydroascorbic acid can likewise be recycled by GSH. Much research has been gathered on the role of Vit E in both hepatotoxicity and the carcinogenic process. Chen and coworkers (1990) fed weanling Sprague-Dawley rats a diet with or without 100 I.U. Vit E supplementation. The Vit E deficient diet resulted in a significant decrease in hepatic levels of Vit C and GSH with a

corresponding increase in lipid peroxidation. Diquat, a known free radical generator, decreased hepatic Vit E and increased lipid peroxidation in Sprague-Dawley rats (Sandy et al., 1987). In a similar fashion, Andersen and Andersen (1993) compared the effects of two antioxidants, α-tocopherol and β-carotene, on lipid peroxidation (LPO) induced by methyl mercuric chloride. CBA mice were fed diets of 10, 100 or 1000 mg/kg high α-tocopherol or β-carotene for four weeks. After the first two weeks, animals from each group were given 40 mg/L of methyl mercuric chloride in their drinking water in addition to the dietary antioxidants. High Vit E (α-tocopherol) reduced LPO caused by methyl mercuric chloride where as low dietary Vit E enhanced the LPO. Interestingly, β-carotene increased LPO in all cases.

The role of Vit E in the carcinogenic process; however, is less clear. There have been both positive and negative correlations with carcinogenesis. An epidemiological study by the Finnish Health Clinic examined a group of 36,265 individuals over an 8 year period. 766 out of 36,265 individuals tested developed some form of cancer (Knekt, 1991). The cancer patients were age and sex matched to controls (no signs of cancer present). A significant decrease in serum Vit E was observed in the cancer patients. Likewise, Gerrish and Gensler (1992) showed Vit E to reduce the incidence of skin cancer in C3H/HeN mice irradiated with Ultraviolet B (UV-B) for 30 minutes a day, 5 days a week. There were three groups of mice: control, 100

U/kg of Vit E and 200 U/kg Vit E. After 31 weeks, the incidence of tumors was 67% in controls, 46% in the 100 U/kg Vit E group and 19% in the 200 U/kg Vit E group. In another study, Vit E, when administered topically, was able to lower the incidence of skin papilloma resulting from DMBA initiation and TPA treatment in female CF1 mice (Perchellet et al., 1985). In this experiment, 40 umole Vit E was applied to mouse skin 5 minutes prior to treatment with 8.5 umole TPA. After 22 weeks, the TPA treated group without Vit E had an average of 13.2 papillomas per animal whereas the Vit E treated group with TPA had only 3.4. This may be a result of Vit E's ability to down-regulate protein kinase C which is specifically activated by TPA rather than Vit E's role as an antioxidant (Mahoney and Azzi, 1988). Likewise, Vit E has been shown to inhibit cell proliferation and enhance transforming growth factor β secretion in human breast cancer cells lines (Charpentier et al., 1993). Whether this growth inhibition was due to Vit E's antioxidant ability or some other factor is yet to be resolved.

In contrast, increased dietary Vit E resulted in an increase in proliferation of mammary tumors in female Balb/c mice (Gerber et al., 1990). Gerber and coworkers transplanted murine mammary tumor (EMT6) into mice on either control or Vit E supplemented diet (0.011%). There was a significant increase in cell proliferation in tumors in Vit E group than in control. In another experiment, Glauert and coworkers (1990) examined the effects of Vit E supplemented diet on ciprofibrate induced hepatic tumors in

female Sprague-Dawley rats. Rats were fed diets of 10, 50 or 500 ppm Vit E with 0.025% ciprofibrate. Rats on higher Vit E diet had an increase number of foci and increased focal volume when compared to lower Vit E diet groups. Furthermore, there was no difference in the level of conjugated dienes or thiobarbituric acid reactive intermediates (both of these are measures of oxidative stress) between the groups. Likewise, Mitchel and McCann (1993) showed Vit E to be a complete tumor promoter in the skin of SINCAR mice. In this study, SINCAR mice were initiated with dimethylbenz-[a]anthacene (DMBA) and treated topically twice a week with either 80 uM of Vit E or 2 uM 12-O-tetradecanoylphorbol-13-acetate (TPA) (a potent skin tumor promoter). After 50 weeks, the TPA group had 100% tumor incidence and the Vit E group had 96% tumor incidence. The only major difference observed was that Vit E did not produce an inflamatory response as did TPA. Thus, Vit E may have a dual role in tumorigenesis. On one hand, it removes the oxidative stress which can lead to the selection of altered hepatic foci; on the other hand, it can protect and promote other types of cancer cells.

Ascorbic acid (Figure 5) has been proposed as a vehicle by which the tocopheryl radical can be reduced and recycled (Chen, 1989). Ascorbic acid, itself, may act as either a pro-oxidant or an antioxidant depending on its local environment and concentration (Bendich et al., 1986). Ascorbic acid once oxidized can then be reduced either by GSH or NADH (Chen, 1989 and Wefers and Sies, 1988). Ascorbic acid is also a cofactor in hydroxylation

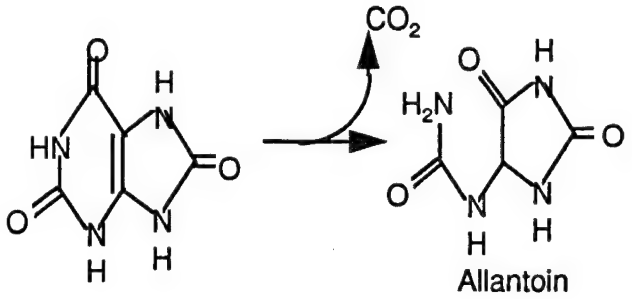
reactions during the synthesis of collagen. It transfers electrons to enzymes providing reducing equivalents during the synthesis of hydroxyproline and hydroxylysine. Surprisingly, unlike UA, man and higher primates are unable to synthesize ascorbic acid. Rats and mice, on the other hand, have the enzyme needed to convert l-gulonolactone to l-ascorbic acid (Marcus and Coulston, 1993). Several human studies have shown a decrease of ascorbic acid during chronic pancreatitis, alcoholism and hypertension (Gut et al., 1994; Lecomte, et at 1994; Tse et al., 1994). Pauling (1991) in a study on the incidence of spontaneous mammary tumors in RIII mice noted a significant delay before the appearance of the first tumor in mice receiving 8.1% dietary Vit C (82.5 weeks for control and 124.9 weeks for high Vit C. In rats, which can synthesize ascorbic acid, Horio et al., (1983) noted an increase in Vit C when polychlorinated biphenyls (PCB), DDT, amionpyrine and chloretone were administered. Vit C, by virtue of its antioxidant capability, has been shown to reduce risk of stomach and esophageal cancer in humans as well as inhibit apoptosis (programmed cell death) in ovarian granulosa rat cells (Sauberlich et al., 1994; Tilly and Tilly, 1995).

Lastly, GSH (Figure 4) is central to the antioxidant defense system of the cell. It can reduce ascorbic acid for reuse via a thiol tranferases and act as a substrate for GSH Px to reduce hydrogen peroxide (Meister, 1992). Furthermore, a reduction in GSH levels with age has been suggested as a mechanism for increased susceptibility to neoplasia (Richie, 1992).

Interestingly, several investigators have found higher levels of GSH in tumors. Wong and coworkers (1994) noted eight time the amount of GSH in human epidermoid carcinomas when compared to normal tissue (24.36 vs 3.04 nmole/mg protein). Likewise, Chang and coworkers (1993) observed twice the GSH levels in squamous cell carcinomas of the cervix as compared to control tissue. This higher level of GSH may be a result of increased synthesis or uptake by the tumors. In support of the latter, Cameron and coworkers (1991) noted an increased uptake of GSH by tumor tissue as compared to normal in F344 male rats when the livers of these rodents were perfused with a GSH solution.

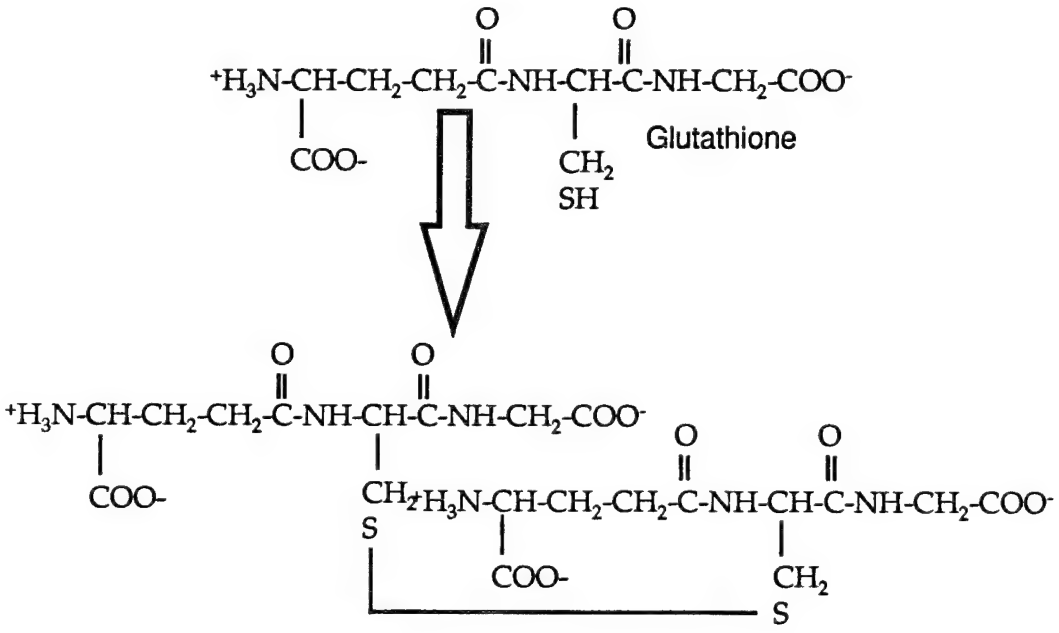
The enzymatic defense systems consist of substances that either directly act to remove free radicals or act to recycle non-enzymatic molecules. The cornerstones of the enzymatic system are three enzymes: SOD, CAT and GSH-Px. SOD takes the superoxide radical and converts it to hydrogen peroxide. There are two forms of SOD: SOD-1, a Cu,Zn form, in the cytosol (McCord and Fridovich, 1969) and SOD-2, a Mn form, in the mitochondria (Weisiger and Fridovich, 1973). CAT converts hydrogen peroxide to water and oxygen and GSH-Px reduces peroxides, both organic and inorganic, using GSH as substrate. GSH-Px also has two forms: a Se-dependent form that can be measured using cumene hydroperoxide as a substrate (Lawrence and Burk, 1976) and a non Se-dependent form. SOD and CAT have been shown to decrease in skin upon treatment with TPA (Reiner, 1991). Also, SOD and GSH-Px have been shown to decrease in hepatocellular carcinomas

(Vo et al., 1988). Furthermore, Jungueira (1986) has shown SOD and CAT to decrease with lindane treatment in rat livers but GSH-Px increases. Cerutti (1991) further states that the ratio of Cu,Zn SOD and CAT are critical in determining the fate of cells exposed to ROS. High SOD levels in cells make them more sensitive to growth inhibition whereas high CAT levels prevent this effect. Thus, changes in cellular enzyme levels may give initiated cells a selective growth advantage. Figure 6 shows how the enzymatic antioxidant defense system works to reduce the pro oxidant state of the cell.



Uric Acid

A. Uric acid and its oxidized form allantoin



B. Glutathione and its oxidized form glutathione disulfide

Figure 4: Reduced and Oxidized forms of Glutathione and Uric Acid

Uric Acid can be converted to Allantoin either by reactive oxygen species or by urate oxidase (in lower species). Likewise, glutathione another cytosolic antioxidant is oxidized to the disulfide when it reacts with reactive oxygen species.

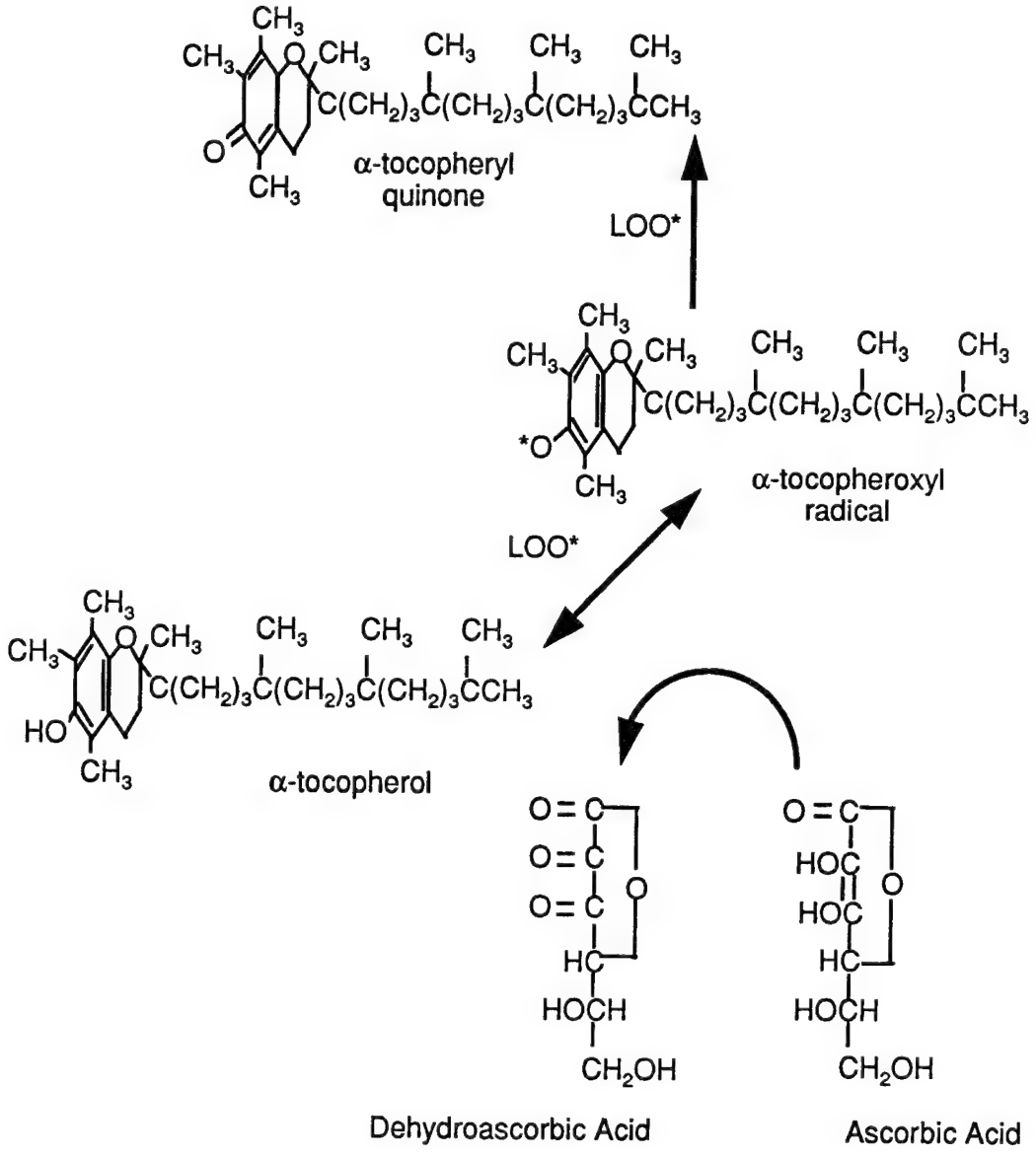


Figure 5: Redox Cyclling of Vitamin E by Vitamin C

α tocopherol (Vitamin E) reacts with lipid peroxide radicals (LOO*) to form the semistable tocopheroxyl radical which can be recycled by ascorbic acid (Vitamin C) or further react with a second LOO* to form α tocopheryl quinone.

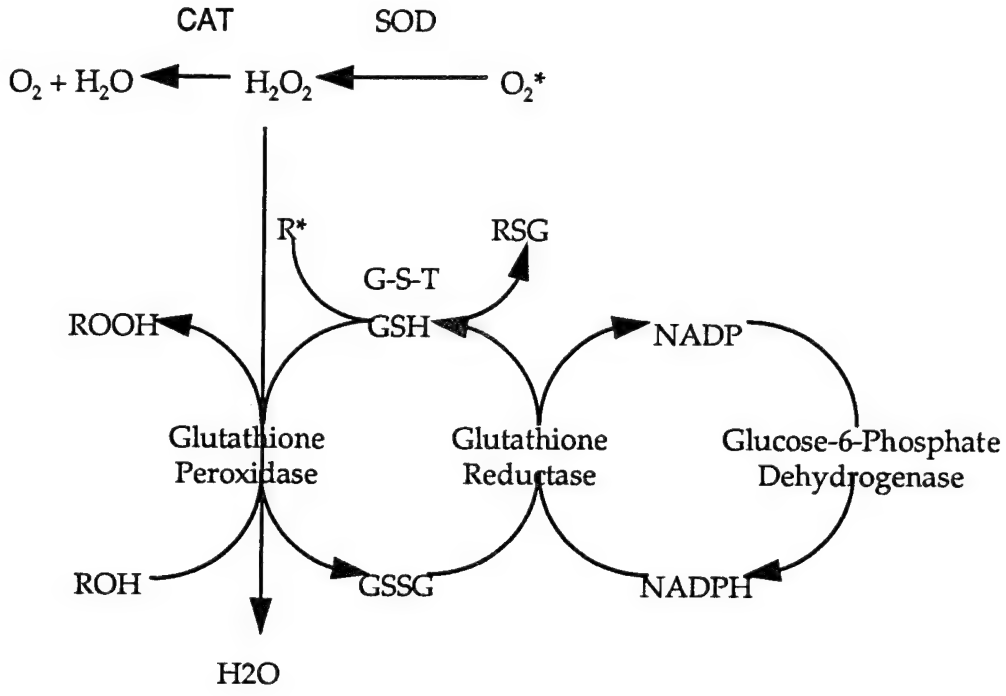


Figure 6: Enzymatic Defense System

The enzymatic antioxidant defense system consist of catalase, superoxide dismutase, glutathione peroxidase, and glutathione S-transferase. Superoxide dismutase converts the superoxide radical to hydrogen peroxide which can then be converted to water either by catalase or glutathione peroxidase. Glutathione S-transferase can convert free radicals to glutathione conjugates. Glutathione reductase is used to recycle glutathione from its oxidized form back to its reduced form and glucose-6-phosphate dehydrogenase uses glucose-6-phosphate to generate NADPH which fuels the glutathione reductase reaction.

Table 1: Studies Examining the Association of Vit E and Cancer

System	Results	Reference
Rats with Vit E deficient diet	decreased Vit C and GSH increased LPO	Chen, 1990
Wistar Rat Chronic Ethanol treatment	decreased serum Vit E	Sadrzadeh et al., 1994
UV-B irradiated C3H mouse skin	Vit E lowered incidence of skin tumors	Gerrish and Gensler (1993)
CBA mice with Mercuric Chloride	high Vit E (1000 ppm) protects against induced LPO	Andersen et al., 1993
Human lymphoma cells	inhibition of TNF-α activation of NF-κB by Vit E	Yulchiro et al., 1993
Mammary tumors in Balb/c mice	increased Vit E in tumors increased proliferation	Gerber et al., 1990
Female Sprague-Dawley rats with ciprofibrate	increased Vit E resulted in increased carcinogenesis	Glauert et al., 1990
Finnish Health Clinic Epidemiological Study	lower serum Vit E yielded higher cancer risk	Knekt, 1991
Male and Female BD-VI rats induced with N-methyl-N-nitro N-nitroguanidine (MNNG)	Vit E decreased incidence of stomach tumors by 50%	Balansky et al., 1986
7,12-dimethylbenz[a] anthracene induced oral tumors in rats	Vit E prevented epidermoid carcinomas	Trickler, 1987
Diquat in Sprague-Dawley rats	decreased Vit E increased LPO	Sandy et al., 1987

C. XENOBIOTICS AND OXIDATIVE STRESS

Many different classes of xenobiotics have shown evidence of inducing oxidative stress (table 2). These can act through several different mechanisms: 1) direct production of free radicals, 2) production of ROS via the cytochrome P-450 system, 3) mitochondria dysfunction, 4) peroxisome proliferation, 5) macrophage activation, or 6) down regulation of antioxidants.

The metabolism of xenobiotics in the cell can be divided into two major categories: Phase I biotransformations and Phase II biotransformations. Phase I reactions convert foreign compounds to more water soluble molecules by oxidation, reduction or hydrolysis reactions. In contrast, Phase II reactions are conjugation reactions (Sipes and Gandolfi, 1991). The major Phase I metabolic system is cytochrome P-450. Cytochrome P-450 consists of NADPH cytochrome P-450 reductase and a heme containing enzyme, cytochrome P450. Cytochrome P-450s are located in the endoplasmic reticulum phospholipid matrix. The cytochrome P-450 system can generate free radicals in the process of normal metabolic processes. These radical species may be ROS or carbon centered radicals such as the trichloromethane radical formed during dechlorination of carbon tetrachloride. The non ROS radicals can react with other molecules to form ROS. One theory of action for the carcinogeniety of xenobiotics involves the biotransformation of the target

compound to a substance with high electrophilic reactivity or free-radical intermediates (Magee et al., 1974; Kehrer, 1993). Other compounds, such as semiquinones and nitroso compounds, can form superoxide radicals by redox cycling in the P-450 system (Figure 7) (Parke and Ioannides 1990). Lubet and coworkers (1989) have shown a strong correlation between inducers of the cytochrome P-450 system and a compound's hepatocarcinogenic ability.

Mitochondria are formed from growth and division of pre-existing mitochondria. They contain their own DNA, mRNA and protein synthesizing system. Unlike genomic DNA, mitochondrial DNA (mtDNA) lack histones which protect DNA from environmental factors. Boveris and coworkers (1972) state that 2% of the oxygen used by mitochondria in cellular respiration results in ROS production. Nohl and coworkers (1989) have shown this ROS production to be increased by TCDD which may in part explain the cytotoxicity of this compound. Based on this evidence, Bandy and Davison (1990) suggested that mitochondrial DNA mutations may produce aberrant mitochondria which reduce oxygen to reactive intermediates. This may then explain a role for mitochondria in aging and cancer. In fact, Sohal and coworkers (1994) have shown mitochondria free radical production to vary inversely with aging. Other xenobiotics have been shown to preferentially react with mtDNA as opposed to genomic DNA. N-(C14)methyl-N-nitrosourea methylates mtDNA 5 times greater than genomic

and cyclophosphamide reacts 100 times greater (Wunderlich et al., 1971; Neubert et al., 1981).

Peroxisomes (microbodies), unlike mitochondria, contain no DNA or ribosomes and are surrounded by a single lipid membrane (Alberts et al., 1989). Peroxisomes are a major site of oxygen utilization where molecular oxygen is used to remove hydrogens from organic substrates. One of the end products of this reaction is hydrogen peroxide. Typically, CAT, which is present in the peroxisomes, reduces the hydrogen peroxide produced to water. However, certain xenobiotics are able to cause peroxisome proliferation. When this occurs, the β-oxidation of fatty acids in peroxisomes may increase 20 to 30 fold while CAT activity increases only 2 fold (Rao and Reddy, 1991). Thus, 10 times more peroxide is generated than there is CAT to remove it. This may place an increased level of oxidative stress on the cell. Many peroxisome proliferators, which are hepatocarcinogens, have been associated with oxidative stress. These include DEHP, [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (WY-14,643) and clofibrate (Tamura et al., 1990; Wada et al., 1992).

Macrophages, known as Kupffer cells in the liver, have been shown to directly produce ROS and cause secondary ROS via the release of TNF-α which can activate nitrogen oxide production (NO) (Nathan et al., 1986). The release of these ROS are essential for tissue maintenance. Kupffer cells in the liver are used to clear particulate and foreign materials from the portal

circulation via phagocytosis. Furthermore, ROS from macrophages are used to combat viruses, bacteria, fungi, tumor cells and other foreign substances. However, these same macrophages, when activated via artificial means, can produce ROS which can be toxic to healthy cells. ElSisi et al., (1993) have shown carbon tetrachloride to activate macrophages. Phenobarbital, acetaminophen, and galactosamine have also been shown to activate Kupffer cells (Laskin et al., 1986; Laskin et al., 1988; Shiratori et al., 1986). Interestingly, Kupffer cells have also been implemented in stimulation of DNA synthesis in hepatocytes after partial hepatectomy (Katsumoto et al., 1989).

Lastly, some xenobiotics have been shown to decrease levels of antioxidants. Jungueira and coworkers (1986) treated 90 day old Wistar rats with lindane by i.p. injection. After 24 hours, both SOD and CAT levels had decreased in a dose responsive matter. Also, SOD and GSH-Px have been shown to decrease in hepatocellular carcinomas (Vo et al., 1988). A similar effect was noted by Reiner (1991) in mouse skin. In this case, treatment of the skin with TPA resulted in decreases of the antioxidant enzymes, SOD and CAT, relative to untreated controls.

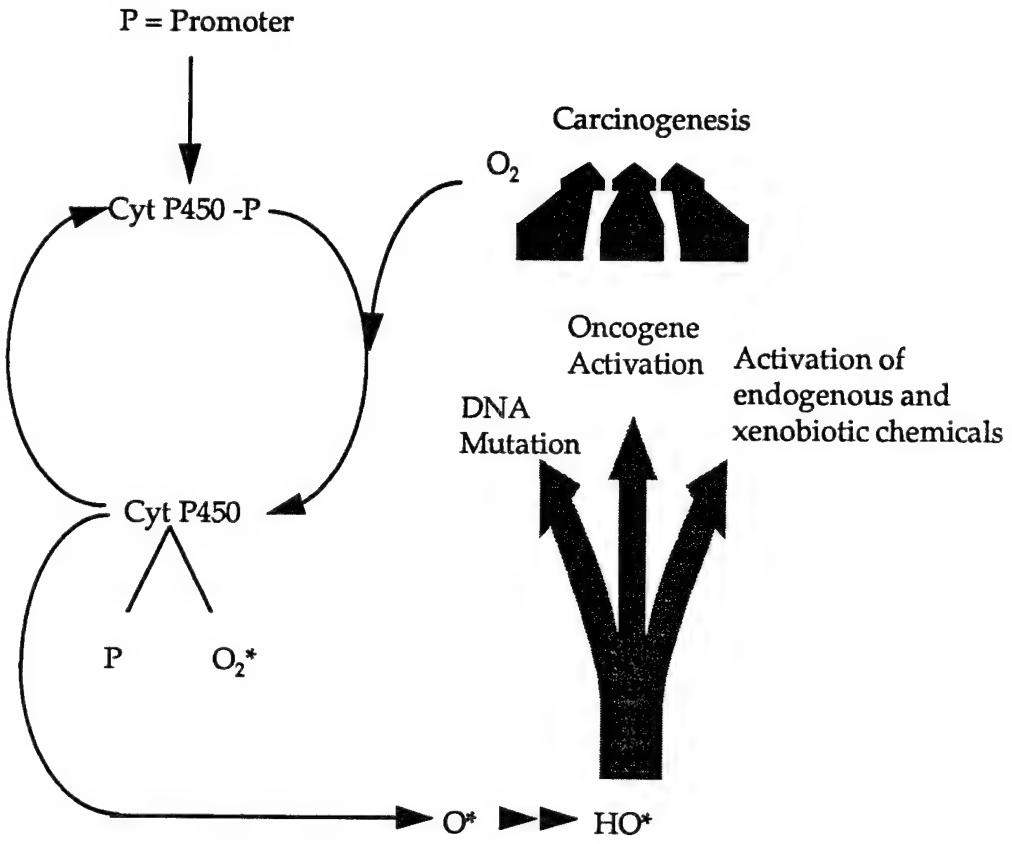


Figure 7: Cytochrome P-450 Futile Cycling

The cytochrome P-450 system can engage in futile cycling in which a chemical agent acts as a substrate for the Cyt P-450 system but is not fully metabolized. Instead it cycles through the Cyt P-450 process resulting in the generation of superoxide. Superoxide can be converted to the more reactive hydroxyl radical and result in DNA mutatioin by oxidizing base pairs, activation of oncogenes such as NF-kB or adduct formation with other compounds which in turn might prove to be carcinogenic.

TABLE 2: Xenobiotics Production of Oxidative Stress

Compound	System	Results	Reference
Benzene	Humans	increased serum LPO decreased GSH Px and SOD	Chen 1992
Lindane		decreased SOD and CAT	Junqueira et al., 1988
Chloro, bromo, and iodobenzene	Sprague-Dawley rat hepatocytes	increased LPO reversed by DPPD	Coleman (1990)
Paraquat and CCl4	hepatocytes	increased free radicals; decreased GJIC	Ruch and Klaunig (1988)
Cocaine	microsomes	increased luminol chemilumi-nescence	Boelsterli et al., (1993)
Nickel	Chinese hamster ovary (CHO) cells	increased oxidation of 2,7-dichlorofluorescin	Huang et al., (1994)
TCDD	female Sprague-Dawley rats	mitochondria LPO	Stohs et al., (1990)
3,4-Catechol estrogen	MCF-7 cells	hydrogen peroxide production	Nutter et al., (1994)
Nafenopin	F344 rat hepatocytes	increased conjugated dienes	Tomaszewski et al., (1990)
pp-DDT	male Wistar Rat	increased LPO (TBARS)	Barros et al., (1994)
Vit E deficient diet	Sprague-Dawley rats	increased LPO	Ono et al., (1986)
Phenobarbital and linoleate	male F344/N rat	decreased Vit E increased PGF2a	Hendrich et al., (1991)

D. CONSEQUENCES OF OXIDATIVE STRESS

ROS can target lipids, proteins, or DNA to produce free radical induced cell injury. The decreased functionality of oxidized proteins and their lost due to proteolysis can effect biochemical and physiological functions (Figure 8). These resulting changes in the cell caused by oxidative stress can have multiple consequences on the cell biology and physiology. Xenobiotic interaction with cells can result in several possible outcomes: 1) damage is not sufficient to cause alteration of cell function, 2) damage is temporary: and the cell can repair the damage and regain normal homeostasis, 3) a sublethal but sustained dose of xenobiotic forces cell into a state of suboptimal functionality, 4) xenobiotic damges the cell but the cell overcompensates for the damage and increases its functionality, or 5) damge results in cell death. Increased DNA synthesis can results from these processes. Cell death can cause regenerative DNA replication while overcompensation of cellular functions may result in a mitotic signaling cascade via increased activation of transcription factors (i.e., AP-1 or NF-κB).

Cell Death

Oxidative stress can trigger cell death either through necrosis or apoptosis. Necrosis is a multistep process leading to cell death (Decker, 1993). It is marked by accumulation of Ca^{2+} and termination of nucleic acid

and protein synthesis. Initially, cell injury may be reversed but under continued xenobiotic treatment it can proceed on to necrosis. This process can be initiated by ROS formation indirectly by Kupffer cells and TNFα or directly by hepatotoxicity with xenobiotics. Apoptosis is a defined chain of events which occurs within the cell to signal its demise. It can increase Ca^{2+} which activates protein kinase C. This, in turn, can phosphorylate G-proteins which increase cAMP (Evans 1993). *bcl*-2 can bind to this G-protein and interrupt this cascade thus preventing apoptosis.

Membrane Damage

MDA is a terminal by-product of lipid peroxidation (Figure 9). Its formation results from the oxidation of polyunsaturated fatty acids (PUFA) by free radical. When oxygen binds to a fatty acid at a carbon-centered lipid radical, the resulting lipid peroxyl radical can further propagate peroxidation chain reactions by removing a hydrogen atom from a near by PUFA. This in turn can result in a molecule of MDA. MDA has been shown to be mutagenic to bacteria and mammalian cells and carcinogenic to rats (Mukai and Goldstein 1975). Furthermore, MDA is capable of forming adducts with DNA (Chaudhary et al., 1994). Lipid peroxidation can result in a loss of cellular homeostasis by changing membrane characteristics. Membrane damage and dysfunction results in loss of calcium and other ion transport systems as well as possible loss of GJIC. This in turn can lead to transcription factor

activation or cell death. Thus, MDA provides a useful tool for the analysis of tissue damage by oxidative stress.

Protein Modification

Pacifici and Davies (1991) hypothesize that modification of a protein by thiol cross-linking resulting from oxidative stress causes hydrophobicity (denaturation). This results in loss of primary, secondary or tertiary structure and allows the protein to be attacked by macrooxyproteinase (MOP). Since liver mitochondria have no CAT, they rely on GSH to combat oxidative stress (Neubert et al., 1962). Some proteins are highly sensitive to changes in cellular thiol levels such as the calcium-dependent ATPase (Bellomo et al., 1983) and thus calcium sequestion can be altered. These oxidized proteins are then susceptible to proteolysis (Davies, 1986). In the extreme case, cell death can occur. Reed (1990) states in his review on calcium and thiols in cellular injury that lipid peroxidation and liver necrosis only occur when GSH levels fall to 10 -15% of their initial levels. The GSH level is then a critical factor in determining the status of oxidative stress in a cell.

DNA Damage

One of the molecular mechanisms of oxygen free radical carcinogenesis is DNA damage, especially formation of oh8dG which is produced by

hydroxylation in the C-8 position of deoxyguanosine (dGuo) residues in DNA by hydroxyl radicals (Breimer, 1990; Kasai et al., 1986) (Figure 10). Its formation in the target organ is related with chemical carcinogenesis (Breimer 1990; Floyd 1990). Interestingly, Hruszkewcyz (1990) reported that DNA itself can act as an antioxidant and be a scavenger for chain breaking lipid peroxidation reactions thus resulting in DNA damage. Finally, oxidative damage to DNA can activate poly(ADP ribose)polymerase. This enzyme utilizes large amounts of NAD to repair damaged DNA which in turn can deplete the cells ATP supply (Kehrer, 1993) (Figure 11). As with LPO and GSH depletion, a loss of ATP can result in impairment of ion transport systems.

The resulting DNA damage from oxidative stress may be in either the genomic DNA or mitochondrial DNA (mtDNA). Richter (1992) reported mtDNA to have 16 times higher basal levels of oxidative damage then genomic DNA in rat hepatocytes. This may be a result of increased ROS production inside the mitochondria or a result of 'naked' DNA in the mitochondria. Furthermore, Shay and Werbin (1992) and Hadler (1989) reported that mtDNA fragments can be inserted into genomic DNA as a result of aging or oxidative damage; thus acting like an oncogenic virus. Bandy and Davison (1990) in a review article cited the ability of mitochondrial mutations to produce changes in cell surfaces which may allow descendent cells the ability to unify and enhance chances for survival and

compete with normal cells for resources. This may in fact explain the tumor promotion ability of many xenobiotics. oh8dG levels thus offer a useful metric of genomic and mitrochondrial DNA tissue damage. Likewise, urinary levels of oh8dG, are a useful non evasive biomarker of DNA damage by ROS (Simic, 1994).

Cellular Homeostasis

Inhibition of GJIC has been implemented in the hepatotoxicity of xenobiotics and the carcinogenesis process (Klaunig et al., 1991 and 1990). The possibility that ROS may have a role in GJIC inhibition was also explored by Ruch and Klaunig (1988). Gap Junctions are cellular organelles located in the plasma membrane. These structures allow for the transfer of low molecular weight materials (1 kDA or less) between the cytoplasm of adjacent cells (Loewenstein, 1979). Each gap junctional plaque consists of numerous small pores, referred to as connexons, that line the pore. One possible function of gap junctions may be to permit the exchange of low molecular weight growth regulating substances between adjacent cells (Loewenstein, 1979). This exchange of chemical information, or GJIC, has been shown to be inhibited by most tumor promoters in a variety of cultured cells (Klaunig and Ruch, 1989). The exact mechanism by which xenobiotics may regulate GJIC is unknown but previous work has shown it to be related

to oxidative stress in cultured hepatocytes (Cerutti 1991, Troll and Wiesner, 1985).

Another mechanism for selective growth by oxidative stress involves activation of oncogenes. Cellular growth may occur when cells are protected against excessive toxicity but when a sufficient oxidant signal remains to activate mitogenic pathways (Cerutti, 1991). This hypothesis was supported by evidence collected by Pronzato (1993) which showed a dose-dependent increase in protein kinase C (PKC) activity with carbon tetrachloride, a known inducer of oxidative stress. However, as the dose was increased, the oxidative stress reduced PKC activity. PKC activity is further potentiated by increased Ca^{2+} levels which also resulted via oxidative stress (Jones, 1983). Interestingly, the activation of the oncogenes AP-1 and NF-κB can be modulated by oxidative stress both directly and indirectly.

AP-1 is a member of a family of proteins that bind to the sequence of TGAGTCA (phorbol ester responsive element) (Radler-Pohl et al., 1993). It consists of two proteins: c-fos and c-jun joined together by a leucine zipper. AP-1 controls genes that are required for cell growth and its activity is increased by proliferating agents. External stimuli can cause activation of existing AP-1 and new synthesis. UV radiation, a proven free radical producer, has been shown to activate the mRNA for c-Jun (Devary et al., 1991). Once activated, it must be deactivated or transformation will result. Radler-Pohl and coworkers (1993) suggest glucocorticoid receptors can act in

the deactivation of AP-1. They found that substitution of three amino acids on the zinc finger regions of the glucocorticoid receptor can prevent its negative interference with AP-1 activity.

Protein Kinase C is required for activation of AP-1 and it has been shown to be activated by hepatic tumor promoting chlorinated hydrocarbons (Moser and Smart, 1989; Kass, 1989). Furthermore, AP-1 has been associated with induction of GST activity. AP-1 can also be induced by Michael reaction centered compounds (i.e., double bound with electron withdrawing groups) (Taladay, et al. 1988). Dieldrin contains such a structure. Recently, it has been shown that AP-1 can be induced by depletion of GSH (Bergelson, 1994). This induction leads to the production of xenobiotic metabolizing enzymes (GSTs, glucuronosyl transferases, and NAD(P)H quinone reductase).

NF-κB has been referred to as the oxidative stress-responsive transcription factor (Schreck et al., 1992). NF-κB is a heterodimer consisting of a 50kD and a 65kD subunit. The activity of the NF-κB dimer is controlled by an inhibitory subunit IκB. In non stimulated cells NF-κB resides in the cytoplasms. It migrates into the nucleus only after the removal of the inhibitory factor IkB. Schreck and coworkers (1992) showed that TNFα was a strong inducer of NF-κB. Earlier work by Baeuerle and Baltimore (1988) provided evidence for the release of IκB from the NF-κB heterodimer in the presence of hydrogen peroxide. It seems quite possible, than, that the redox

state of the cell may act as a second messenger system in the modulation transcription factors. Indeed, Abate and coworkers (1990) found that the DNA binding of Fos-Jun (AP-1) heterodimer was modulated by the reduction or oxidation of a single cysteine residue in the DNA binding domain of the two proteins. By using thioredoxin, an enzyme which catalyzes the reduction of cysteine residues, improved DNA binding of AP-1 was possible in an isolated reaction mixture.

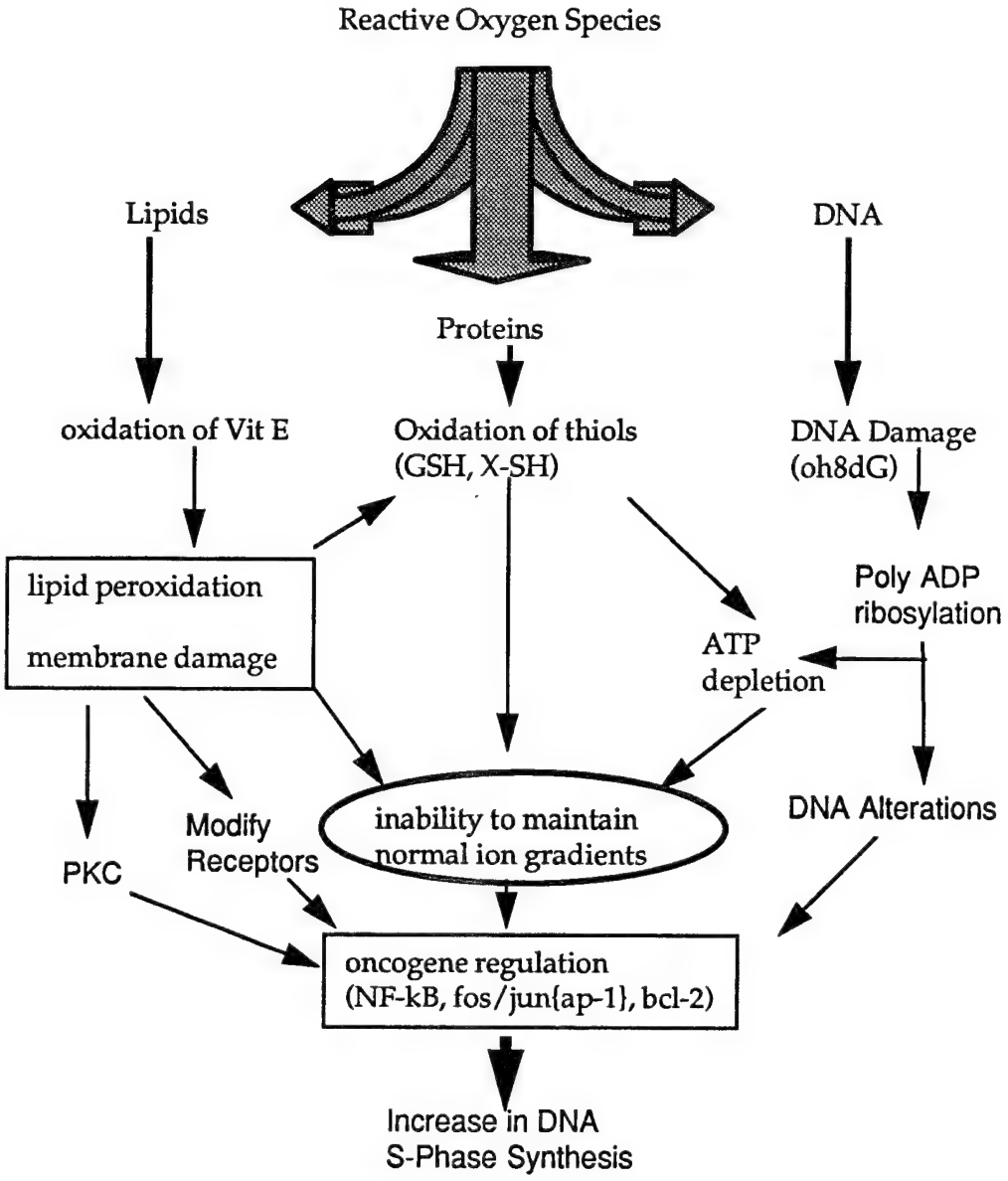


Figure 8: Consequences of Oxidative Stress

Oxidative stress can result in lipid peroxidation and depletion of Vit E. This in turn can result in activation of protein kinase C or modifiy proteins resulting in transcription factor activation. Likewise, depletion of GSH can lead to loss of cellular ionic homeostasis which can result in cell death if severe enough or activation of transcription factors. Finally, damage to DNA will result in activation of poly ADP ribosylation which is utilized in DNA repair. This in turn can lead to depletion of ATP and effect ATP driven ionic pumps.

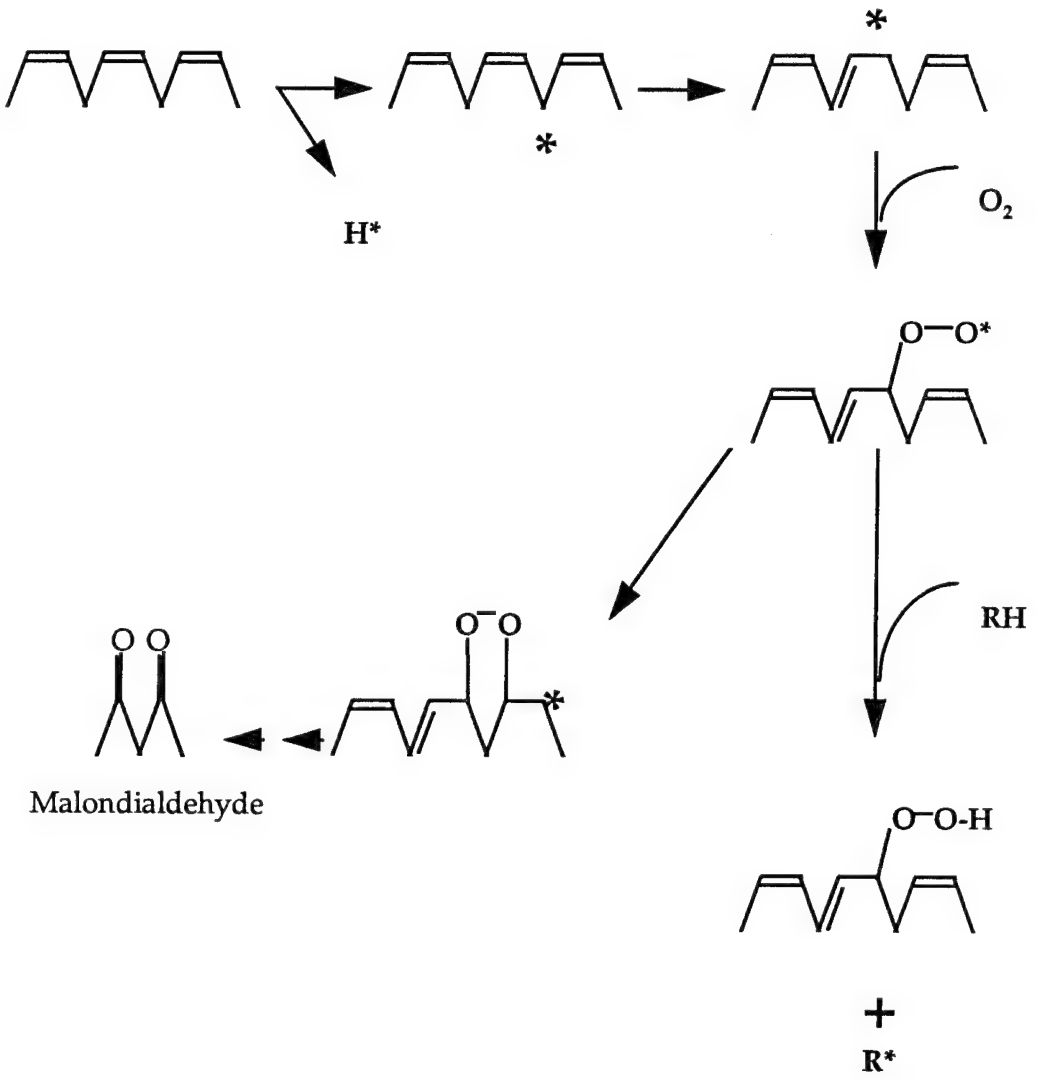


Figure 9: Lipid Peroxidation: Formation of Malondialdehyde

Polyunsaturated Fatty Acids (PUFA) can form free radicals which may react with molecular oxygen to form lipid peroxide radicals. These lipid peroxide radicals can react with hydrogen groups on other compounds (i.e. Vitamin E) to form stable lipid peroxides or form malondialdehyde which can then be measured in tissue and urine.

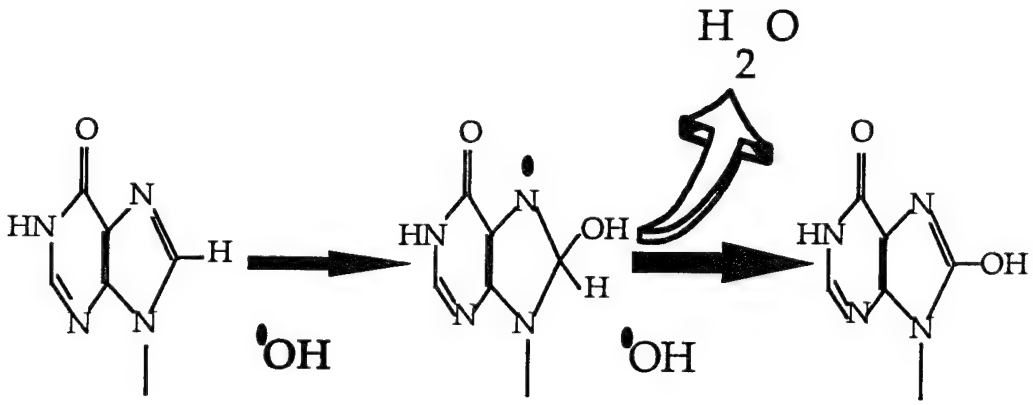


Figure10: 8-hydroxy-2'-deoxyguanosine Formation

The hydroxyl radical can react with 2'-deoxyguanosine to form the 8-hydroxy-2'-deoxyguanosine product which is detected by HPLC analysis.

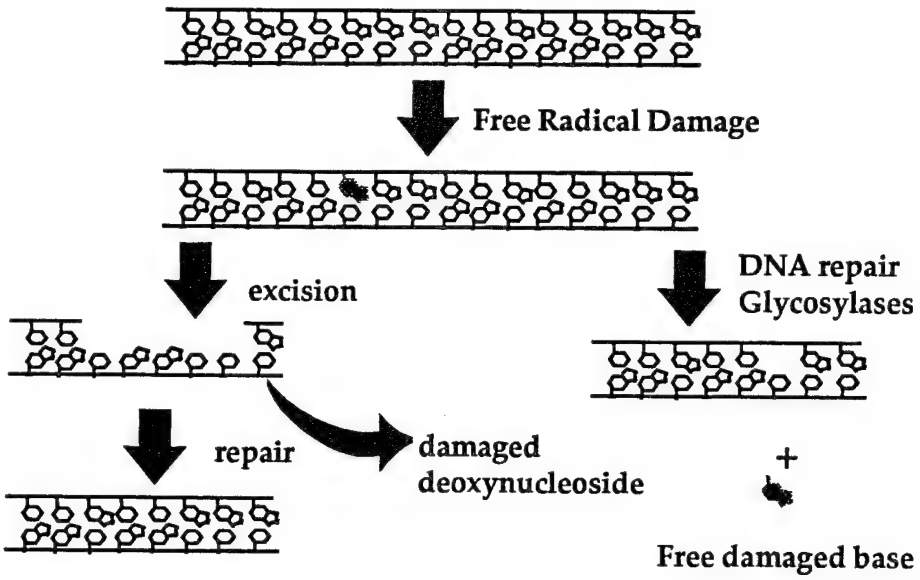


Figure 11: Repair of Oxidative Stress induced DNA Damage and Repair

When a nucleotide is damaged by oxidative stress, it can either cause a potential mismatch during DNA replication, repaired and replaced by excision repair of excised by Glycoslases leaving a gap. The freed nucleotide can be detected in the urine of mammalian species.

E. 0XIDATIVE STRESS AND CANCER

Cancer

The current theory of cancer recognizes three stages: initiation, promotion, and progression. Initiation of a cell results in a mutational change in cellular DNA, usually through direct interaction of a chemical (or its metabolites) with the DNA. Tumor promotion occurs when agents (promoters) increase the tumorogenic response of an initiating carcinogen when administered after the initiator (Klaunig et al., 1990, Klaunig, 1991). This can occur by several possible mechanisms: 1) inhibition of the normal apoptosis pattern of hepatocytes (Wyllie et al., 1992), 2) induction of DNA synthesis in initiated vs normal hepatocytes or 3) the resistance hepatocyte model in which initiated cells are more resistant to the cytotoxic effects of xenobiotics than normal cells (Solt and Farber, 1976). Tumor progression involves the development of malignant neoplasms from benign lesions.

Role of Xenobiotics in Cancer

In conjunction with this three phase model of carcinogenesis, xenobiotics have been classified as either genotoxic or non-genotoxic. Genotoxic chemicals are those which cause a genetic mutation and are involved in the initiation phase of cancer. At a high enough dose, some genotoxic chemicals can also be promoters. Once a mutation has occurred, it

is necessary that cell division occurs to fix the mutation in the genomic DNA. This cell division may occur by natural cell turnover, tissue growth (as seen after a partial hepatectomy) or accelerated cell division brought on by xenobiotics or mitogens. Nongenotoxic carcinogens on the other hand, do not cause initiation but are involved in the clonal expansion of neoplastic or preneoplastic cells (Yamasaki, 1988). Non genotoxic carcinogens have been shown to function through a variety of different mechanisms such as receptor activation, increased DNA synthesis, decreased apoptosis, inhibition of GJIC or a change in gene expression. Still some other chemicals may act by altering the cell membrane, thus interfering with regulatory factors from normal to spontaneously altered cells (Williams, 1981).

ROS have been proposed as one mechanism by which xenobiotics may realize their carcinogenicity; they are believed to be involved in aging and different stages of carcinogenic process (Trush and Kensler, 1984, Sun, 1990; Cerutti and Trump, 1991; Borek, 1991; Frenkel, 1992; Bankson, 1993; Clayson et al., 1994; Schwartz, 1993). One theory of action for the carcinogeniety of xenobiotics involves the biotransformation of the target compound to a substance with high electrophilic reactivity or free-radical intermediates (Talalay et al., 1988). As mentioned above, free radicals can create a state of oxidative stress in the hepatocytes leading to lipid peroxidation, DNA damage or modulation of the antioxidant defense system of the cell. The resulting oxidative stress forces a variety of changes upon the

hepatocyte. These changes may be genotoxic, as in DNA strand breaks and mutations caused by oxidative damage to DNA, or non-genotoxic changes such as oncogene and enzyme induction (Kehrer, 1993). ROS can either directly cause DNA breakage or result in an increase in pH and Ca^{2+}. This rise in pH and Ca^{2+} can also activate endonucleases resulting in DNA strand breaks. Furthermore, the activation of critical sulfhydral groups on protein kinase C (PKC) due to modulation of cellular homeostasis can cause its activation even without an increase in calcium. PKC has been implemented in AP-1 induction which is associated with cell proliferation (Radler-Pohl et al., 1993). Besides, activating oncogenes that stimulate proliferation, oxidative stress can also activate oncogenes which inhibit apoptosis. A decrease in apoptosis by the oncogene bcl-2 or an increase in proliferation by AP-1 or NF-κB could both result in preferential growth of initiated cells over normal cells.

Nongenotoxic hepatocarcinogens induce a variety of changes in the liver after only a few weeks of treatment, long before tumors are detectable. These changes include hypertrophy as a result of enzyme induction such as cytochrome P-450, increased mitosis and hepatomegaly (Schulte-Hermann, 1987). These early changes are considered to be adaptive in nature due to the increased metabolic demand placed on the liver by the xenobiotic (Schulte-Hermann, 1974). The hypertrophy and hyperplasia may act to increase the total P-450 content to handle the increased metabolic demand.

However, cell proliferation and DNA synthesis are critical to all three stages of cancer (Columbano et al., 1991). Even though many nongenotoxic chemicals increase DNA synthesis well in advance of tumor formation, it is quite possible that this early synthesis burst is critical in the cancer process. Ames and Gold (1992) contend that spontaneous mutations occurring naturally in cells may become fixed and create initiated cells. These initiated cells can then later be selected for promotion by xenobiotics.

Stimulation of DNA synthesis in subchronic studies is a predictive tool to evaluate the carcinogenicity of xenobiotics (Busser and Lutz, 1987). Other investigators have reviewed the connection between cell proliferation and cancer (Butterworth and Goldsworthy, 1992). While some disagreement exist as to the relevance of early DNA synthesis to tumor formation (Melnick 1992), a wide variety of xenobiotics have been shown to induce DNA S-phase synthesis in subchronic studies prior to the appearance of preneoplastic foci. In table 3 and 4, a comparison has been made to show a connection between xenobiotics which induce DNA synthesis and which have also been shown to induce oxidative stress. The peroxisome proliferators di(2-ethylhexyl)-phthalate (DEHP), Nafenopin and clofibrate all induce DNA synthesis in F344 rats (Smith-Oliver and Butterworth, 1987; Reddy and Rao, 1977; Reddy and Qureshi, 1979). The barbituate, phenobarbital, has been shown to induce DNA S-phase synthesis in both mice and rats (Stevenson et al., 1995). The inorganic compound, lead nitrate, induced S-phase synthesis in male

Wistar rats (Columbano et al., 1987). Chlorinated compounds, such as carbon tetrachloride, ethylene dibromide, DDT and TCDD, all produce an early increase in DNA S-phase synthesis (Columbano et al., 1987, Busser and Lutz, 1987; Lucier et al., 1991). Interestingly, these same compounds have been associated with changes in the oxidative stress level within the liver. Lipid peroxidation has been observed with DEHP, Clofibrate and DDT in rats (Tamura et al., 1990; Barros et al., 1994). Nafenopin increased conjugated dienes in rats and TCDD produced superoxide anion formation in mice (Lake et al., 1991; Alsharif et al., 1994). Likewise phenobarbital has been shown to decrease hepatic Vit E levels while carbon tetratchloride increased urinary MDA (Hendrich et al., 1991; Bagchi et al., 1993). Finally, Ledda-Columbano (1994) has shown lead nitrate and ethylene dibromide to increase TNFα. TNFα has been shown, itself, to promote free radical formation. Thus, a diverse group of carcinogens may very well function through a mechanism involving oxidative stress.

In 1976, Solt and Farber proposed the resistance hepatocyte model in which initiated cells are more resistance to the cytotoxic effects of xenobiotics than normal cells. One way in which initiated cells may express this resistance is in their ability to proliferate in an oxidative stress environment. This may be accomplished via a superior defense against ROS or activation of oncogenes such as AP-1, enzyme induction (i.e., SOD/CAT) (Cerutti and Trump, 1991) or via activation of an oncogene that inhibits apoptosis such as

Bcl-2 (Ikegaki et al., 1994). Thus, as stated earlier, spontaneous mutations fixed by early burst of DNA synthesis may result in promotable foci.

Table 3: Hepatocarcinogens which have been shown to induce DNA S-phase synthesis in subchronic studies.

Compound	Animal Species	Reference	Oxidative Stress Observed	Animal Species	Reference
WY14,643	F344 rats	Reddy et al., 1979; Marsman 1988	increase lipofuscin	F344 rats	Wada et al., (1992)
DEHP	B6C3F1 male mice; F344 rats	Smith-Oliver and Butterworth 1987; Marsman 1988	lipid peroxidation	male F344 rats	Tamura et al., (1990)
Perchloroethylene	B6C3F1 male mice	Schumann et al., 1980	metabolize to trichloro-ethylene(see below)		
Nafenopin	male F344 rats	Reddy and Rao 1977	increase conjugated dienes	Sprague-Dawley rats	Lake et al., (1991)
Clofibrate	male F344 rats	Reddy and Qureshi 1979	lipid peroxidation	male F344 rats	Tamura et al., (1990)
2-Nitropropane	F344 male rats	Cunningham and Mattew 1991	8-hydroxydeoxy-guanosine	Sprague-Dawley male and female rats	Guo et al., (1990)
Gasoline	B6C3F1 male and female mice	Tilbury et al., 1993	contains benzene which can induce LPO	human study	Chen 1992
TCDD	Sprague-Dawley female rats	Lucier et al., 1991	superoxide anion formation	C57Bl mice female Sprague-Dawley rats	Alsharif et al., (1994) Bagchi et al.,

Table 4: Hepatocarcinogens which have been shown to induce DNA S-phase synthesis in subchronic studies.

Compound	Animal Species	Reference	Oxidative Stress Observed	Animal Species	Reference
α-HCH	Wistar female rats	Rolf-Schulte Herman 1974	lipid peroxidation	male Wistar rats	Junqueira et al., (1991)
DDT	Osborne-Mendel male rats	Busser and Lutz (1987)	lipid peroxidation	male Wistar rats	Barros et al., (1994)
Lead nitrate	Wistar male rats	Columbano et al., 1987	TNFα increased	rats	Ledda-Columbano et al., (1994)
Carbon tetrachloride	Wistar male rats	Columbano et al., 1987	urinary MDA increase	female Sprague-Dawley rats	Bagchi et al., 1993
Ethylene Dibromide	Wistar male rats	Columbano et al., 1987	TNF α increased	rats	Ledda-Columbano et al., (1994)
Phenobarbital	B6C3F1 male mice and F344 male rats	Stevenson et al., 1995	decreased α-tocopherol	female F344 rats	Hendrich et al., (1991)
Trichloroethylene	B6C3F1 male and female mice	Dees and Travis 1993	increased lipid peroxidation	male Sprague-Dawley rat hepatocytes	Kefalas and Stacey (1989)
Furan	B6C3F1 mice and male F344 rats	Wilson et al., 1992	decreased GSH	F344 rat hepatocytes	Carfagna et al., (1993)
Dieldrin	B6C3F1 male mice	Stevenson et al., 1995			

F. DIELDRIN

History

This research initiative will examine the role of ROS in the selective action of dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-exo-1,4-endo-5,8-dimethanonapthalene) in mouse livers using the B6C3F1 mouse and the F344 rat. Dieldrin was first produced in the United States in 1950 (US Tariff Commission, 1951). Its parent compound, aldrin, quickly degrades to dieldrin by hydrolyzation of the epoxide group. Dieldrin was used as an insecticide, primarily for treatment of soil, to control termites. By 1973, the US Environmental Protection Agency listed dieldrin as a toxic pollutant. However, like many other first generation pesticides, it persists in the environment for long periods of time. The values range from 0.001 to 1 ppm in the environment (Table 5). Thus, even though dieldrin is no longer used commercially in the United States, it still represents a potential hazard due to its persistence in the environment and at various storage sites. Dieldrin was chosen for this study because it is typical of a wide variety of organochlorinated compounds which are hepatocarcinogens in mice but not the rat.

Literature Review

While it has been shown to increase tumor incidence in mice, no correlation has been made with increase cancer incidence in rats or humans (de Jong et al., 1991; Thorpe et al., 1973, Deichmann et al., 1970). Table 6 provides a summary of selected dieldrin studies. Davis and Fitzhugh (1962) conducted a two year study using C3HeB/Fe mice which were placed on 10 ppm dieldrin diet. The average survival time of dieldrin treated mice decreased 8 weeks from controls and there was a significant increase in tumor incidence of dieldrin treated mice. Tennekes and coworkers (1982) observed similar results in the CF1 mouse with the median time to tumor incidence being lowered with increasing concentration of dieldrin in the diet. Mice were given either 0, 0.1, 1, 2.5, 5, 10 or 20 ppm dieldrin in their diet for 150 weeks; the mean time to tumor formation was 129, 119, 116, 106, 92, 64 and 48 weeks respectively. The 0.1 ppm dieldrin group in the male CF-1 mice was not significant from control but all other groups were. Since the age specific tumor incidence at 0.1 ppm dieldrin was almost identical to the spontaneous tumor incidence seen in controls, it suggests that dieldrin does not act as a complete carcinogen but only a promoter. This statement is supported by two other studies. The first by van Ravenzwaay and coworkers (1988) examined nuclear polyploidization and tumor incidence in dieldrin treated CF-1 mice. They showed that liver tumor formation was associated with a constant level of polyploidization. Dieldrin increased the level of

polyploidization so that it occurred at earlier time points in a dose-dependent manner. Since polyploidization is an age dependent process, they concluded that dieldrin may act by advancing the biological age of CF-1 mouse livers. Thus, dieldrin did not cause initiation, it only caused spontaneous tumors to occur at earlier time points. The second study by Bauer-Hofmann and coworkers (1992) showed that dieldrin increased the number of c-Ha-ras wild type focal liver lesions in male C3H/He mice but not the mutated c-Ha-ras type. The c-Ha-ras wild type oncogene is indicative of spontaneously initiated tumors suggesting that dieldrin is not an initiator. Oxidative stress has been implicated in the aging process (Cutler, 1984). Indeed, De and Darad (1991) have shown an age dependent decrease in Vit E and Vit C in Wistar rat serum with age and a decrease in both hepatic and serum GSH levels. It may then be possible that the promotion of spontaneously initiated tumors by dieldrin may be a result of premature aging of the mouse liver due to prolonged oxidative stress.

In a multi-species study by Wright et al., (1972), dieldrin-induced alterations in subcellular structure were reversible in rats, mice and beagle dogs. In all three species, microsomal protein levels increased and an increase in smooth endoplasmic reticulum was observed. The rhesus monkey used in this study showed only a slight increase in microsomal protein levels with no increase in liver size. An increase in tumor incidences in these species was only observed in mice Walker et al., (1969). Hutterer (1960)

initially showed dieldrin to increase the smooth endoplasmic reticulum and cytochrome P-450 activity. While this was true for both mouse and rat livers only the mouse produced tumors on chronic dieldrin treatment (Walker, 1977; Thorpe and Walker et al., 1973; Tennekes et al., 1982; Deichmann et al., 1970). Work by Baldwin and Robinson (1972) and Hutson (1976) showed that the mouse was slower to metabolize dieldrin and that it did not form the pentachloroketone metabolite which predominated in rats. Interestingly the mouse did produce several polar metabolites not seen in rats. When Obenholser (1977) blocked the P-450 metabolisms of dieldrin in rats using SKF-525A new metabolites were reportedly formed. This suggest that a difference in the way each of these species metabolize dieldrin may contribute to dieldrin's selective carcinogenic effect in mice.

Dieldrin Pharmacokinetics and Metabolism

The selective action of dieldrin in mice may be a result of several factors: 1) pharmacokinetics (storage and excretion), 2) metabolism or 3) target tissue uptake. Studies by Baldwin and Robinson (1972) and later by Hutson (1976) showed several differences in all three of these factors.

The CF1 mouse retains dieldrin to a much greater extent than the CFE rat. Furthermore, the amount of unchanged dieldrin in rat urine is much greater than the mouse while unchanged dieldrin in mouse feces is greater than the rat. This suggests different metabolic pathways may exist

between these species. Figure 16 shows the common metabolic products of dieldrin metabolism. Metabolic studies show the mouse excretes only minute amounts of a dieldrin metabolite, pentachloroketone (PCK). Interestingly, the mouse has higher levels of PCK in the liver than the rat; suggesting it is disposed of via an alternate metabolism. This may account for the unidentified polar metabolites found in mouse urine which are not in rat urine. In addition to these differences, the mouse retains 10 times as much dieldrin and PCK in its fat and 8 times as much in its liver as the rat.

Suppose, however, that it is not the metabolic products, storage or excretion of dieldrin that results in its specific action in mice but the process of forming these products. Hutson (1976), in the closing statement of his paper on the comparative metabolism of dieldrin in mice and rats suggested that the selective action of dieldrin "...may be the operation of a common biotransformation mechanism (e.g. mono-oxygenation) rather than the formation of a specific metabolite, that causes the toxic effect observed in the CF1 mouse." If this biotransformation mechanism creates ROS, it may then account for dieldrin's selective action in mice.

The species difference in this rodent model has been the subject of several investigations. Parke and Ioannides (1990) contend that the mouse due to its smaller size and higher metabolism may exist in a more stressed condition. This is, in part, supported by Sohal (1990) and Ku et al., (1993) who compared mitochondria activity among several species. The mouse

produced higher levels of superoxide than the rat. Parke and Ioannides further state that the mouse can engage in futile cycling in which a compound can continually cycle through the cytochrome P-450 system and generate ROS. He suggested that the P-450 IIB system which can use aldrin (the parent compound of dieldrin) as a substrate can engage in futile cycling. The P-450 IIE system (ethanol metabolism) and the P-450 IV (peroxisome proliferator metabolism) can also generate free radicals in this fashion. Thus, the generation of free radicals through activation of the P-450 system may be a common route of tumorgenicity for many structurally unrelated compounds.

Evidence for Oxidative Stress

Recent work by Stevenson et al., (1995) has shown dieldrin-induced S-phase synthesis in mouse livers to be reversed by increasing the level of Vit E in the diet. Vit C, on the other hand had just the opposite effect. As mentioned earlier, Vit C can act as both a antioxidant and pro-oxidant. Interestingly, no difference was observed in hypertrophy of centrilobular hepatocytes or the induction of hepatic mixed function oxidase activity. This suggests that Vit E is acting through its antioxidant capabilities and not by reversing the induction of mixed function oxidase activity by dieldrin. In addition, pilot studies ran with dieldrin show its inhibition of GJIC to be specific to mouse hepatocytes (Bachowski et al., 1995). Rat hepatocytes are

unaffected. Furthermore, this inhibition of GJIC in mouse hepatocytes can be reversed by the antioxidants: Vit E and trolox. Inhibition of GJIC in primary culture of rodent hepatocytes correlates well with the hepatocarcinogenicity and hepatotoxicity of the compound (Klaunig et al., 1991 and 1990). These two markers of hepatocarcinogens (GJIC and subchronic induction of DNA S-phase synthesis) both suggest that dieldrin may in part be acting by creating a state of oxidative stress in mouse hepatocyte.

This species difference is not unique to dieldrin but applies to a variety of xenobiotics. The mouse is much more sensitive to many carcinogens than is the rat (Tomatis et al., 1973). Perchloroethylene, methylene chloride, chlordane, heptachlor and dieldrin (NTP, 1986; NTP, 1988; Hutson, 1975; Baldwin and Robinson, 1972; Williams and Numato, 1984) are all carcinogens in mice but not rats. The exact cause of these differences is, as yet, not well defined. Green (1990) suggests differences in the GSH metabolic pathway to be a chief mediator of carcinogenicity. Parke (1990), on the other hand, stresses the ability of P-450 metabolism and futile cycling. Both of these pathways play a role in the level of oxidative stress in the hepatocytes.

Interestingly, several other halogenated hydrocarbons and hepatocarcinogens have been connected to oxidative stress. Stoh (1990) showed TCDD to induce LPO and DNA damage. Colemam (1990)

demonstrated an increase in LPO of rat hepatocytes exposed to chloro, bromo and iodo- benzene. This increase was not seen when hepatocytes were treated in the present of the antioxidant DPPD (diphenyl-p-phenylenediamine). Izushi (1990) showed heptachlor, a structurally similar compound to dieldrin to increase LPO in mouse livers. HCH, Carbon tetrachloride, hexachlorobenzene, dieldrin and TCDD induced lipid peroxidation in female Sprague-Dawley rats (Goel et al., 1988). DDT caused lipid peroxidation in male Wistar rats (Barros et al., 1994). Lindane produce lipid peroxidation and reactive oxygen species in rats (Hassen et al., 1991; Bagchi et al., 1993).

This data then suggest that dieldrin's tumorigenicity may in fact be due to an increased level of oxidative stress in mouse hepatocytes. In fact, Goel (1988) and Kohli (1977) found no increase in LPO by dieldrin in rat livers. This fact when compared to Stevenson's work suggested that the species difference seen in mice and rats with respect to dieldrin may be a result of increased oxidative stress in mouse livers. Examination of dieldrin-induced oxidative damage and its effect on selective parts of the antioxidant system will be the main thrust of this initiative.

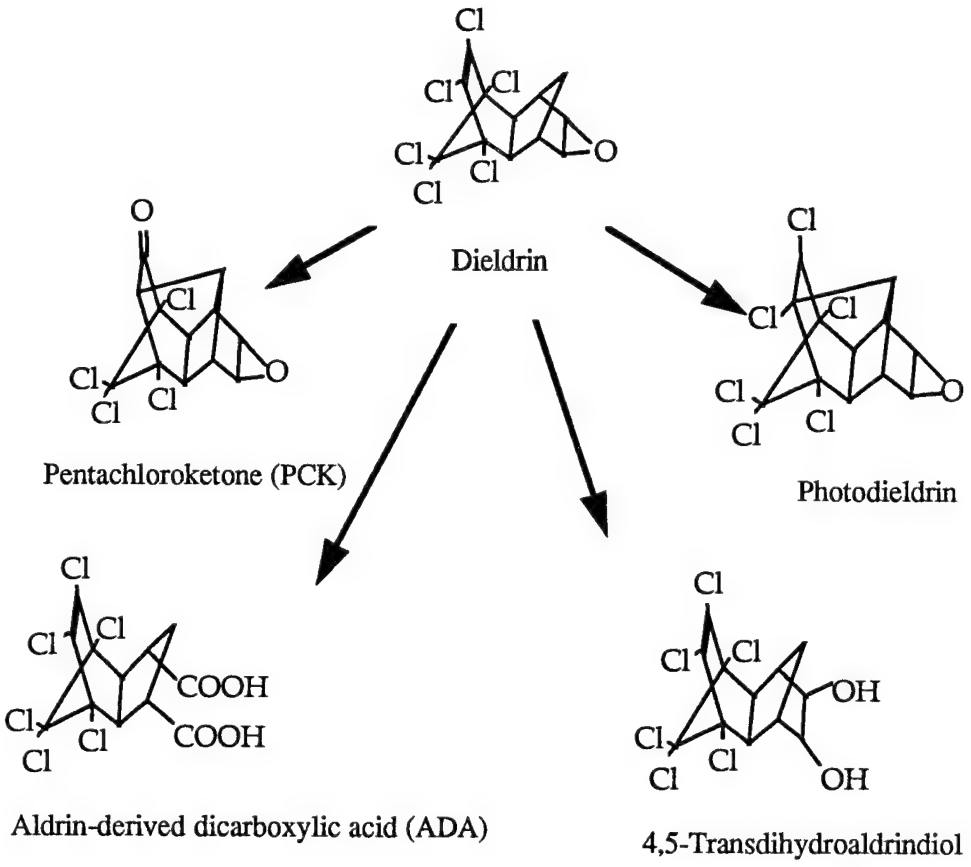


Figure 12: Dieldrin Metabolites

Metabolic studies show that while the mouse retains more pentachloroketone than the rat, it excretes less. Other common products in the mouse and rat which have been identified are aldrin-derived dicarboxylic acid, photodieldrin and the 4,5-transdihydroaldrindiol.

Table 5: Dieldrin in the Environment

Dieldrin Concentration	Location	Reference
1.18ppm (13 years after last use)	cranberry bog soil	Deubert and Zuckerman, (1969)
0.05ppm	carrots grown in dieldrin soil	Sand et al., (1972)
0.16ppm	aquatic insects	Hannon et al., (1970)
0.002-6.7ppm	fish	IARC Monographs, (1974)
0.6-4.3ppm	bird livers	IARC Monographs, (1974)
0.001-0.03ppm	human milk	Curley and Kimbrough, (1969)
0.72-1.1 mg/man/day	exposed workers	Hayes and Curley, (1968)
0.3ng/L	drinking water	Bevenue et al., (1972)

Table 6: Dieldrin Carcinogenesis and Related Studies

Rodent	Dosing	Results	Reference
C3HeB/Fe mice	10ppm (1 yr) control	38/218 (tumor incidence) 77 wks to tumor formation 9/217 (tumor incidence) 89 wks to tumor formation	Davis and Fitzbhugh, (1962)
CF1 mice	dose response	age-specific tumor incidence: rate of formation of tumors does not change with dieldrin but they occur at earlier time points	Tennekes et al., (1982)
Rat liver		no change in LPO	Goel et al., 1988
Sprague-Dawle female rats	5mg/kg	increased SER;P-450	Hutterer et al., (1960)
CF1 mice	10 ppm	100% tumor incidence male mice by 17 months (control 0%) 100% tumor incidence female mice by 26 month (Control 36%)	Thorpe and Walker, 1973
Wistar Rat	30mg/Kg BW	increased P450 decreased LPO	Kohli, 1977
CFE Rats	0.1, 1 and 10ppm for 2 years	no difference intumor incidence	Walker et al., (1969)
Osborne-Mendel rats	20,30 and 50 ppm for 20 months	no tumors observed	Diechmann et al., (1970)
B6C3F1 mice and F344 rat	0, 0.1, 1, 10 ppm	induced DNA synthesis in mice not rats	Stevenson et al., (1995)

MATERIALS

A. ANIMALS

F344 rats, C3H mice, C57Bl mice and B6C3F1 mice were obtained from Harlan-Sprague Dawley, Inc. (Indianapolis, Indiana). Five mice or two rats were housed in each plastic cage and lived under standard conditions: 22±2°C, a relative humidity of 50±10 %, and 12 hr light and dark cycle in animal room. All animals were fed with NIH-07 diet in pelletized form and deionized water *at libitum* and quarantined for 1 wk. All of the procedures described were conducted with the approval of the Animal Care and Use Committee of Indiana University School of Medicine.

B. CHEMICALS

Compound	**Abbreviation**
Acetic acid	HOAc
Acetonitrile, HPLC grade	
Alkaline Phosphatase	
Ammonium Phosphate, HPLC grade	$NaNH_4PO_4$
l-Ascorbic Acid	Vit C
Bovine Serum Albumin	BSA
Cacodylic Acid	$C_2H_7AsO_2$

Compound	Abbreviation
Chloroform, ACS	CCl_3H
Creatinine Kit	
Creatinine Color Reagent	
Acid Reagent	
Sodium Hydroxide	
Creatinine Standard	
Creatinine Standard	
Cupric Sulfate	$CuSO_4$
2'-deoxyguanosine	dGuo
Dexamethasone	DEX
Dieldrin (99% pure)	Dieldrin
Dichloromethane, HPLC grade	CCl_2H_2
2,3-Dihydroxybenzoic Acid	2,3-DHBA
Dimethyl Sulfoxide	DMSO
2,4,-dinitrophenylhydrazine	DNPH
Dulbecco's Modified Eagle's Medium	DMEM/F-12
(DME) and HAM's nutrient mixture	
F-12	
Ethanol-200 proof	EtOH

Compound	Abbreviation
Ethylene glycol-bis-(B-aminoethyl ether)-N,N,N',N',-tetraacetic Acid	EGTA
Ethylenediaminetetraacetic Acid	EDTA
Ethyl Ether	
Fetal Bovine Serum, Characterized	FBS
Formaldehyde	
Gentamicin Solution	
Gentistic Acid, Sodium Salt Hydrate	2,5-DHBA
Glucose-6-Phosphate Dehydrogenase	G-6-PD
Glutathione	GSH
Hank's Balanced Salts Solution	HBSS
Hexane (85% n-hexane)	HEX
Hydrochloric Acid (33%)	HCl
Hydrogen Peroxide (30%)	H_2O_2
(N-[2-Hydroxyethyl]peperazine-N'-[2-ethanesulfonic acid]) sodium salt	HEPES: $C_8H_{17}N_2O_4SNa$
7-hydroxy-2-phenoxazone	Resorufin
Resorufin Benzyl Ether	Benyl Resorufin
Resorufin Ethyl Ether	Ethyl Resorufin
Resorufin Methyl Ether	Methyl Resorufin

Compound	Abbreviation
Isoamyl Alcohol	
L-15 Medium	L-15
LD-L 50 reagent	
Magnesium Chloride	$MgCl_2$
Mercuric Chloride	$HgCl_2$
Methanol, HPLC grade	MeOH
B-Nicotinamide Adenine Dinucleotide Phosphate	NADPH: $C_{21}H_{26}N_7O_{17}P_3Na_4$
N-(1-naphthylene)ethyl-enediamine dihydrochloride	$C_{12}H_{14}N_2$ 2HCL
Nuclease PI	
Pentane	
Perchloric Acid	
Phosphoric Acid	
Potassium Chloride	KCl
Potassium Permanganate	$KMnO_4$
Potassium Phosphate Dibasic Trihydrate	K_2HPO_4 $3H_2O$
Protein Assay Reagent Biorad Coomassie Blue	

Compound	Abbreviation
Proteinase K	
Pyrogallol	$C_6H_6O_3$
B-Resorcylic Acid	2,4-DHBA
Ribonuclease A Type XII-A from Bovine Pancrease	
Salicylic Acid	SA
Sodium Acetate, Trihydrate	NaOAc
Sodium Bicarbonate	$NaHCO_3$
Sodium Chloride	NaCl
Sodium Citrate, Trisodium Salt Dihydrate	
Sodium Nitrite	$NaNO_3$
Sodium Pentobarbital	
Sodium Phosphate dibasic	Na_2HPO_4
Sodium Phosphate monobasic	NaH_2PO_4
Sucrose	
1,1,3,3,-Tetramethoxypropane	
2-Thiobarbituric Acid	$C_4H_4N_2S$
α-Tocopherol	Vit E
α-Tocopherol Acetate	

Compound	Abbreviation
Tricholoracetic Acid	
TRIZMA Base	Tris
Uric Acid	UA
Sodium Dodecyl Sulfate	SDS

C. EQUIPMENT

Centrifuges

Beckman J2-M1 with JA 14 and JA 18.1 rotors

Centra-4B IEC microcentrifuge

Beckman L8-78 with TY 50.2 R1 rotor

Cell Culture and Tissue care and storage

Cell Culture Hoods: Steril-Gard Hood Baker Company, Inc.

Refrigerator/freezer: Kenmore 18

So-lo Deep Freeze: Environmental Equipment

Incubator, cell culture: ESPEC BNA-211

Jung Histo Embeddor

Reichert Jung 2030 biocut

Fisher Scientific LX-120 tissue processor

Fisherbrand Superfrost/Plus Microscope slides

HPLC

Waters 600 E System Controller with 700 satellite WISP autoinjector. An IBM 433DX/D PS value Point computer workstation with Millenium 2010 version 1.10 (1992) software by Millipore was used to process data relayed form a BAS CC-5/LC-4C EC detector and Waters 484 UV detector via a SAT/IN Bus Analog/Digital Converter.

Spectrometers

ROCHE COBAS Mira-S transfer analyzer

Shimatzu UV Spectrometer with IBM 286 computer and software

Perkin Elmer 650-10S Fluorescence spectrophotometer with 150 xenon power supply and Hitachi Model 056-1001 chart recorder

D. CELL CULTURE AND RELATED SUPPLIES

Percision Water Bath (GCA corp)

Master Flex Perfusion Pump (Cole Palmer)

Meyer N-EVAP Evaporator

Organomation tygon tubing size 14

Gram-atic Balances (FISHER)

925 pH/ion Meter pH meter (FISHER)

pH electrode (CORNING)

pH paper pHydrion 1 to 2.5

pHydrion 3.0 to 5.5

60 x 15 mm sterile dishes (CORNING)

100 x 20 mm sterile dishes (CORNING)

1.5 ml eppendorfs (FISHER)

2 ml centrifuge tubes (FISHER)

Serum collection vials

Tissue cassettes

2 ml Cyrotubes

Masterflex 7521-40 Peristaltic Pump (Cole Palmer)

Masterflex 7518-10 Peristaltic Pump Head (Cole Palmer)

Acrocap 0.2 μm #4480 Filters (Gelman Sciences)

25 mm 0.2 μm #4612 SuporAcrodisc Syringe Lock Filters (Gelman Sciences)

60 cc Luer-Lok #9663 Syringe (Becton Dickinson)

E. COMPUTER SOFTWARE

AS/486 IBM compatible computer with Microsoft Word version 6.0, Microsoft Excel version 5.0 and Microsoft Powerpoint version 4.0 (1994) by Microsoft Corporation was used for data processing and report generation.

Macintosh Quadro 950 with Sigma Plot Scientific Graph System version 4.11 (1991) by Jandel Corporation for graphic presentation and SuperANOVA (1991) by Abacus Concepts, Inc. for statistical analysis was used for data analysis.

METHODS

A. HEPATOCYTE ISOLATION

Mouse and rat hepatocytes were isolated by *in situ* portal vein perfusion according to Klaunig et al., (1981) and Klaunig and Baker (1994). Mice or rats were anesthetized with sodium pentobarbital at 2.5 g/kg. The abdominal and thoracic cavities of the anesthetized animals were then exposed and loose ligatures of suture were placed around the superior vena cava above the diaphragm and around the portal vein approximately 5 mm from the liver. A small incision was made in the portal vein below the ligature and a cannula was inserted while perfusate flowed from the cannula. The cannula was secured in the portal vein with the ligature and the vena cava ligature was then tied off. Both perfusates were pre-heated to 42°C for 90 minutes to prevent formation of air bubbles in line during perfusion. The first perfusate solution was sterile calcium and magnesium free Hank's balanced salt solution (HBSS) (Sigma H-2387) containing 0.5 mM EGTA and 0.05 M HEPES (pH=7.2) at 42°C. The flow rate was kept at 3 ml/minute for the mouse or 5 ml/minute for the rat. The perfusate was permitted to flow out a cut in the inferior vena cava made below the kidneys. The vena cava was periodically clamped and released above the cut causing the liver to swell and collapse during the perfusion. The initial perfusion with the Hank's balanced salt solution lasted 15 minutes. Next, the liver

was perfused with sterile Leibovitz's L-15 media (Sigma L-4386) containing 1mg/ml glucose and 0.5 mg/ml collagenase (pH = 7.2) at 42°C. After perfusion with collagenase solution, the liver was removed to a sterile 100 mm petri dish containing 15 ml collagenase solution. The hepatocytes were removed from the connective tissue stroma by gently repipeting until a homogenous suspension occurred. The cells were filtered through a sterile 25 micron meshed screen and centrifuged at 500 x g for 5 minutes. The top layer was suctioned off using a Pasteur pipette. The resulting cell pellet was resuspended in 40 ml DMEM/F-12 media (pH=7.2) (Sigma D-8900) and recentrifuged. Again, the top layer was suctioned off using a Pasteur pipette. The resulting cell pellet was resuspended in 40 ml DMEM/F-12 media (pH=7.2). The number of viable hepatocytes, determined by trypan blue dye exclusion, were counted with a hemocytometer. Hepatocyte viability was always greater than 88%.

B. HEPATOCYTE PRIMARY CULTURES

Cells were plated at a density of 33,000 cells/cm^2 onto either 60 or 100 mm rat tail collagen-coated culture dishes. The plating media consisted of DMEM/F12 media supplemented with insulin (5 μg/ml), gentamycin sulfate (50 ng/ml), dexamethasone (0.8 μg/ml: stock solution 4 mg/ml 95% ethanol) and 5% fetal bovine serum. Cells were then incubated in a 5% carbon dioxide incubator set at 37°C and 95% humidity. After 4 hours the media was

changed. Media was changed every 24 hours thereafter. Cells were dosed with xenobiotics at each media change.

C. PREPARATION OF RAT TAIL COLLAGEN

Rat-tail collagen was prepared by the method of Ehrmann and Gey (1956). The tails of 5 month old F344 Fisher rats were removed at the time of sacrifice. Tails were stored in freezer at -30°C until needed. Two slits were made longitudinally along the length of the frozen tails and the skin removed. The four bands of collagen fibers were removed and allowed to air dry. Collagen was solubilized by soaking collagen fibers in a 1:1000 (v/v) acetic acid solution (100 ml for 1 g collagen). Fibers were left in solution for 72 hours at 4°C with intermittent mixing. The viscous gel was then centrifuged at 10,000 x g for 1 hour and the top layer removed. This top viscous layer was then diluted 1:10 with deionzed water, pH to 7.4, autoclaved and stored in refrigerator at 4°C until needed.

D. CYTOTOXICITY ASSAY: LACTATE DEHYDROGENASE (LDH) RELEASE

Cells for LDH determination were plated at a density of 33,000 cells/cm^2 onto 60 mm collagen coated dishes. Media was changed after four hours and cells were dosed. At 24, 48 and 72 hours 100 µl aliquots of media were removed and filtered through a 25 micron nylon mesh screen to remove

cellular debris. After the 72 hour sampling, 500 µl of 10% triton were added to each dish. Each dish served as its own control. Cells were placed in incubator for additional four hours and media was again sampled. LDH activity was analyzed spectrophotometrically using a COBAS Mira S transfer analyzer with Sigma LD-L reagent. The mean LDH activity in cell free media was also determined from triplicate dishes. The percent of total LDH released for each test culture was then determined as follows:

$$\%LDH = \frac{(\text{sample - media blank})*100}{(\text{triton sample - media blank})}$$

E. DETECTION OF REACTIVE OXYGEN SPECIES

High-Performance Liquid Chromatography (HPLC) was used to detect ROS formation in dieldrin treated B6C3F1 and F344 microsomal fractions according to the method of Floyd et al., (1984) except salicylic acid was used to detect ROS formation (Grootveld and Halliwell, 1986). Salicylic acid forms several products from its reaction with the hydroxyl radical: catechol, 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) (Figure 13). Of these products, only the 2,3-DHBA and 2,5-DHBA

are formed in sufficient quantities for detection by HPLC. 2,5-DHBA can be formed by metabolism of salyiclic acid in the Cyt P-450 system. 2,3-DHBA is the only product formed exclusively by reaction with the hydroxyl radical in amounts which can be measured accurately.

1. Microsome Preparation

Male B6C3F1 mice or male F344 rats were anaesthetized with sodium pentobarbital (2.5 g/kg). The abdominal and thoracic cavities of the anesthetized animals were then exposed. A syringe filled with cold phosphate buffer saline (PBS) solution (136 mM Sodium Choride, 2.68 mM Potassium Choride, 9.6 mM Sodium Phosphate Dibasic, 1.47 mM Potassium Phosphate Monobasic pH=7.6) was then inserted into the inferior vena cava. Three milliliters of PBS solution were flushed through the liver to remove blood. The liver was then removed and rinsed in cold PBS. The livers (~2 g of liver used per preparation) were then placed in two volumes (w/v) of cold sucrose-TKM buffer (0.25 M sucrose, 80 mM Tris-base, 5 mM $MgCl_2$-$6H_2O$ and 2.5 mM KCl (pH to 7.4)). The mixture was homogenized using a Potter-Elvehjem Tissue Grinder with PTFE pestle (Fisher Scientific Co.) for 10 seconds. Homogenate was centrifuged on a Beckman J2-MI Centrifuge using a JA-14 rotor at 10,000 x g for 10 minutes at 4°C. The supernatant was placed in a fresh centrifuge tube and centrifuged at 15,000 x g using a JA-14 rotor for 15 minutes at 4°C. Supernatant was removed to a fresh tube and

centrifuged at 100,000 x g using a TY-50.2 RI rotor for 60 minutes on a Beckman L8-70 Ultracentrifuge at 4°C. The supernatant was discarded and the microsomes gently rinsed with sucrose-TKM buffer. Next, the microsomes were resuspended in 5 ml of sucrose-TKM buffer. The resulting suspension was divided into 0.5 ml aliquots and frozen at -80°C until needed.

2. Characterization of Microsomes

The protein level in the microsomal preparations was determined with the Bio-Rad Protein Assay system based on the shift of Coomassie Brilliant Blue G-250 from 465 to 595 nm. The Coomassie blue dye binds mainly to basic and aromatic amino acid residues, especially arginine. Absorbance of sample was recorded at 595 nm using bovine albumin as a standard.

The cytochrome P-450 content on the microsomes was determined by the method of Burke et al., (1985) and Nerurkar et al., (1993). Three ether's of resorufin were used to characterize the activity of the microsomes: benzyl ether resorufin for cyt P-450 2B, methyl ether resorufin for cyt P-450 1A and ethyl ether resorufin for cyt P-450 1A1. Resorufin [Sigma R-3257] was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 3 ng/ml for use as standard. Resorufin benzyl ether [Sigma R-441], resorufin ethyl ether [Sigma R-352], and resorufin methyl ether [Sigma R-351] were dissolved in DMSO to a final concentration of 0.5 mM. Standard and ethers were stored at -30°C in the dark until needed.

The assay was run on a 650-10S Fluoresence spectrophotometer with a 150 xenon power supply manufactured by Perkin Elmer. The excitation wavelength was 530 nm, emission wavelength was 585 nm and slit width equaled 2 mm. A Hitachi chart recorder, Model 056-1001, set at a chart speed of 5 mm/minute and voltage of 10 mV was used to record data. The PBS buffer was preheated to 37°C in a water bath. A standard curve was then prepared using 25, 20, 15 and 10 µl of 3 ng/ml stock resorufin in 1 ml buffer. The reaction mixture for microsome analysis consisted of 1 ml buffer, 10 µl resorufin ether, 10 µl of 2 mM NADPH and 5 µl of microsomes. Upon addition of microsomes to reaction mixture, mixture was vortexed and emmission was recorded for 12 minutes. Results were reported as nM/min/mg protein.

3. Aromatic Hydroxylation Assay for Microsomes

To a 1.5 ml eppendorf tube were added 50 µl 5 mM Salicylic Acid, 10 µl 25 mM $FeCl_3$/EDTA, 10 µl 2 mM NADPH, 10 µl 5 units glucose-6-phosphate dehydrogenase, 100 µl Buffer 1 (5 mM Glucose-6-phosphate in sucrose-TKM buffer), 50 µl xenobiotic, and 0.5 mg microsome. Total volume was adjusted to 250 µl with sucrose-TKM buffer. Microsomes were added last. Mixture was vortexed gently and placed in 37°C water bath for 30 minutes. After incubation, reaction was stopped by adding an equal volume (250 µl) of 10% trichloroacetic acid. Mixture was centrifuged at 12,000 x g using a JA 18.1

rotor for 10 minutes and immediately analyzed on HPLC. Samples were kept on ice until analysis. The sucrose-TKM buffer consisted of 0.25 M sucrose, 80 mM Tris-base, 5 mM $MgCl_2$-$6H_2O$ and 2.5 mM KCl (pH to 7.4). Figure 14 illustrates the reaction system for the microsome aromatic hydroxylation assay. Glucose-6-phosphate dehydrogenase and glucose-6-phosphate were used to generate NADPH to fuel the Cyt P-450 system.

Xenobiotic Dosing: Dieldrin was prepared as a 100 mM solution in DMSO. This stock solution was diluted 1:40 to yield a 250 μM solution. 50 μl of this solution was added to reaction mixture to yield a final concentration of 50 μM dieldrin. Final DMSO concentration in reaction volumn was 0.05%. For other concentrations of dieldrin, DMSO final concentration was maintained at 0.05%.

4. Aromatic Hydroxylation Assay for Hepatocytes

Cells were isolated and plated out on 60 mm culture dishes at a concentration of 33,000 cells/cm^2. After a two hour attachment period, the media was changed and the cells were dosed with a 10 mM salicylic acid solution (37°C) prepared in DMEM/F-12 medium supplemented with insulin (5 μg/ml), gentamycin sulfate (50 ng/ml), dexamethasone (0.8 μg/ml: stock solution 4 mg/ml 95% ethanol) and 5% fetal bovine serum. Cells were then incubated in a 5% carbon dioxide incubator set at 37°C and 95% humidity.

After 1 hour, the salicylate media was removed and the cells were washed twice with Dulbecco's phosphate buffer saline solution with glucose (DPBS/G) (0.2 g KCl, 0.2 g KH_2PO_4 8 g NaCl, 2.16 g Na_2HPO_4 $7H_2O$ 0.5 g glucose per liter, pH = 7.2). Next, 500 µl of DPBS/G containing the dieldrin dose (in DMSO to yield final DMSO concentration of 0.05%) were applied to the cells. Cells were placed in the incubator for 1 hour. Cells were removed from incubator and 500 µl of a 10% trichloroacetic acid solution were added. Cells sat on ice for 15 minutes. Next, a rubber policeman was used to remove cells from dish. Homogenate was transferred to a clean eppendorf tube and centrifuged at 12,000 x g. 250 µl of supernatant were removed for HPLC analysis.

5. HPLC Analysis Conditions

The HPLC system consisted of a Waters 600E pump with a Waters 700 Satellite WISP autoinjector controlled by a Waters Millennium 2010 software package using an IBM 486 computer. The chromatography system consisted of three Waters Nova-Pak C18 columns, 4 µ, 8 x 100 mm in a Radial-Pak cartridge guarded by a Nova-Pak C18 Guard-Pak Insert. The column was eluted with a 30 mM sodium citrate solution (pH to 4.65 with acetic acid) at a flow rate of 1.3 ml/min. 2,3 dihydroxybenzoic acid and 2,5 dihydroxybenzoic acid were detected on a CC-5/LC-4C Ameperometer Detector from BAS System set at 0.1 µA range, +800 mV potential and 0.1 filter. The two

adducts eluted at ~ 20 and 24 minutes respectively. Salicylic Acid was detected on a Waters 484 Tunable Absorbance Detector set at 296 nm with a elution time of ~75 minutes. Standard curves are shown in figure 15. Standards were made fresh each day in a 5% trichloroacetic acid solution. The final concentration of standards for injection was 2 ng/ul. 5 and 10 µl of each standard were injected. A typical chromatograph of a microsomal sample and a cell culture sample are shown in figures 16 and 17. Figure 18 shows a typical chromatograph for salicylic acid. 100 µl of unknowns were injected for analysis. The run time for each sample was 90 minutes and samples were kept in freezer until analysis time.

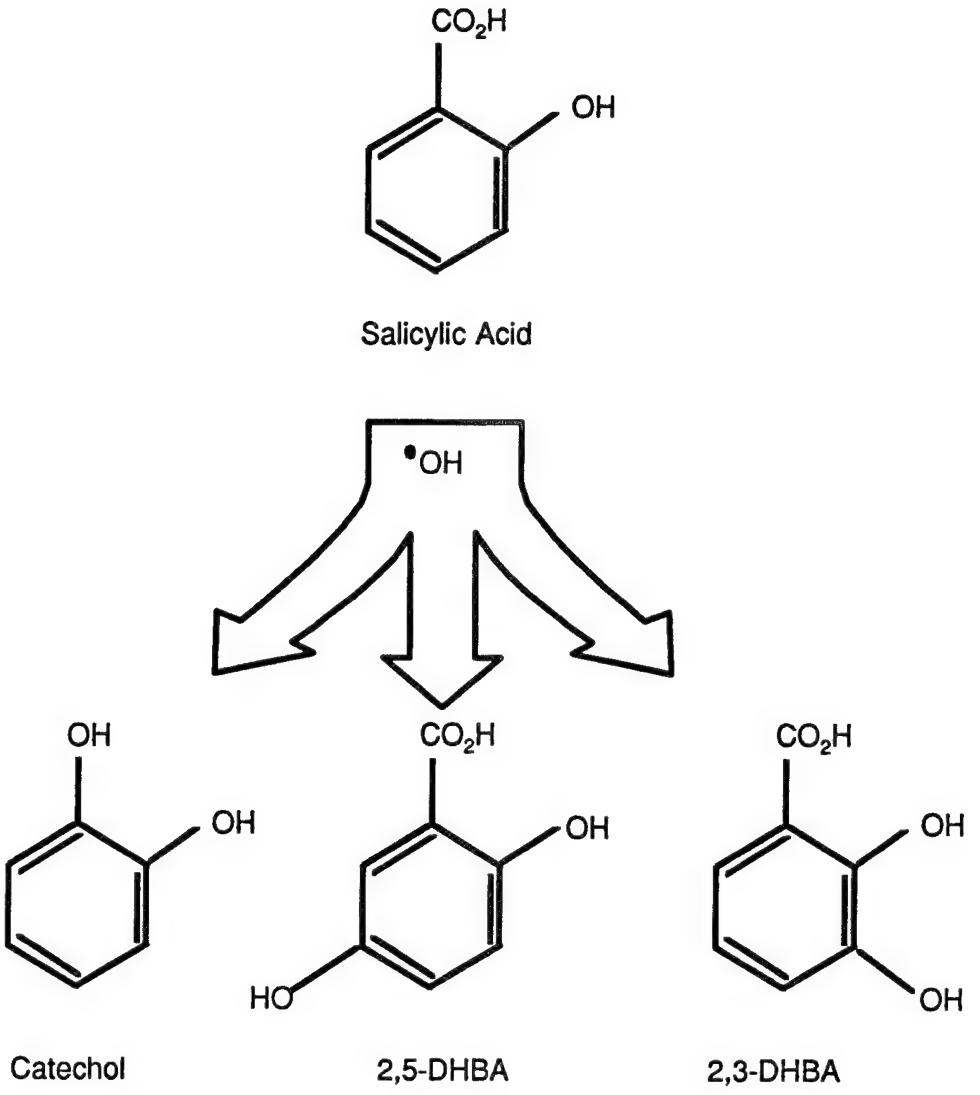


Figure 13: Hydroxyl Radical Products of Salicylic Acid

The Reaction of the hydroxyl radical with salicylic acid can yield three products. Two of them, Catechol and 2,3-dihydroxybenzoic acid (2,3-DHBA), are exclusively formed during the reaction of salicylic acid with the hydroxyl radical. The catechol is a minor product, therefore, the 2,3-DHBA is determined in the aromatic hydroxylation assay. The other product, 2,5-dihyroxybenzoic acid (2,5-DHBA) can also be formed by direct metabolism of salicylic acid with the cytochrom P-450 system.

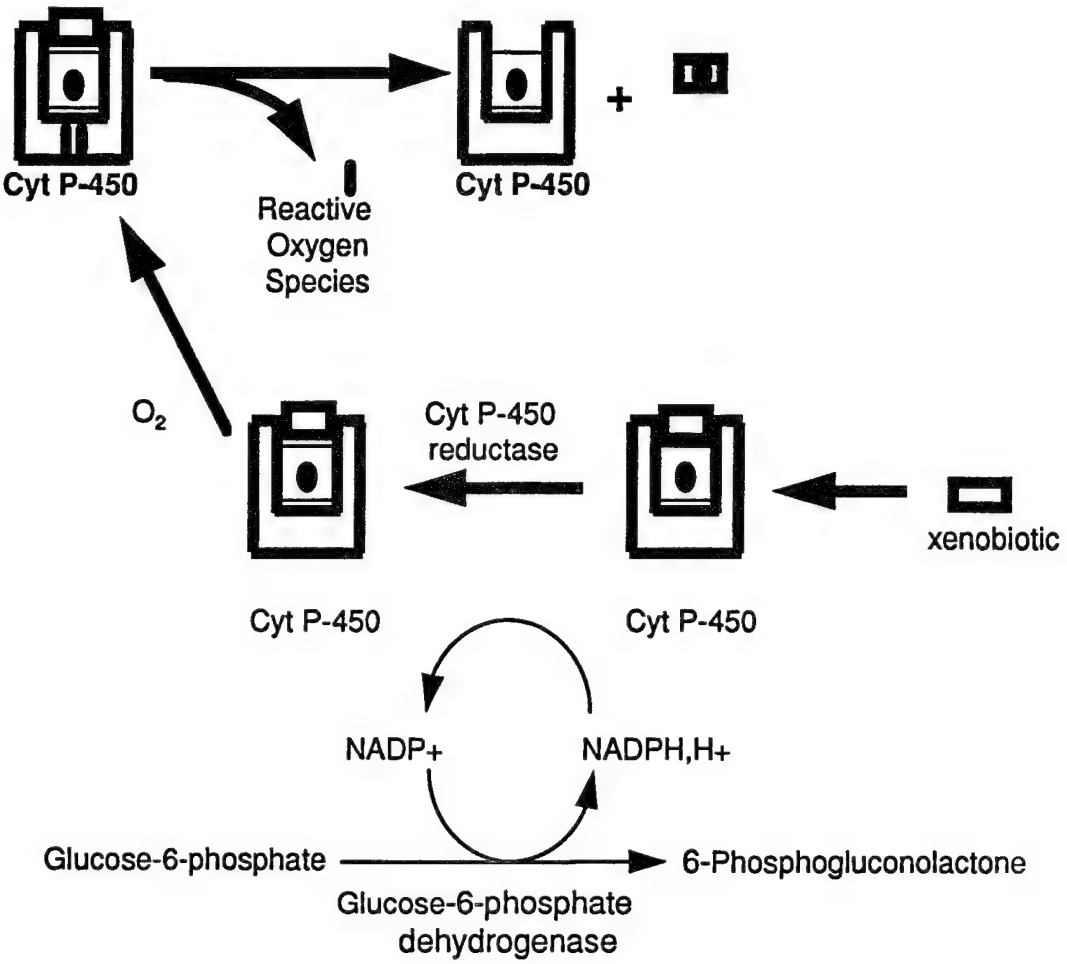
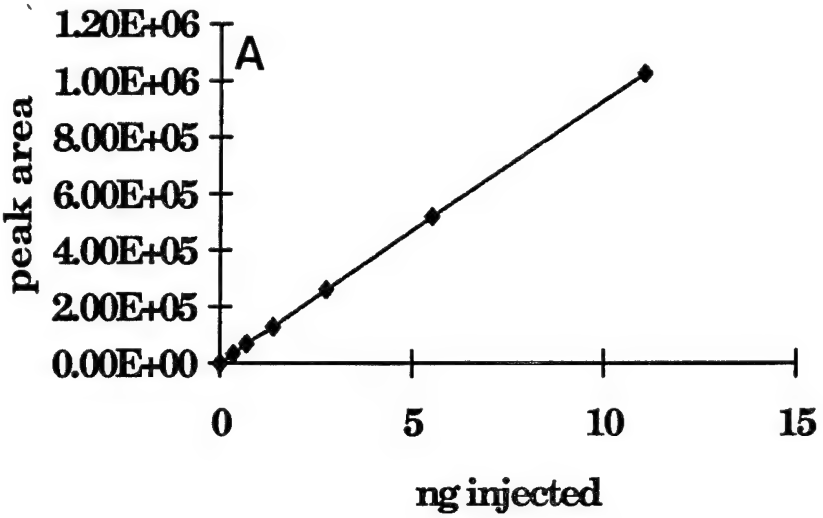


Figure 14: Microsome Reaction System for ROS Detection Schematic of Cyt

P-450 reaction to generate reactive oxygen species. A xenobiotic acts as substrate for the Cytochrome P-450 system. Cytochrome P-450 reductase transfers electrons to the Heme group. Next molecular oxygen is utilized as a second electron donor. The xenobiotic is released in an oxidized form while the molecular oxygen is released as a free radical.



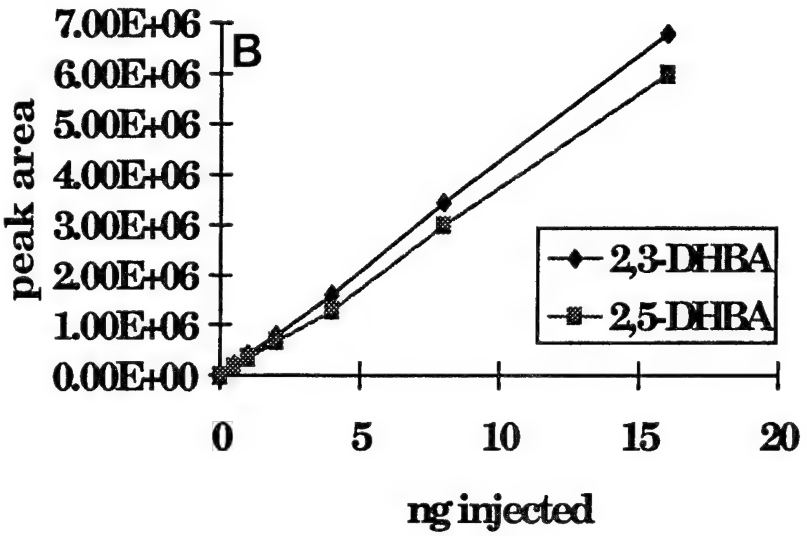


Figure 15A and 15B: Calibration curves for salicylic acid (panel A) and 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) (panel B). Standards were run at the start of each experiment and at the conclusion. r^2 values for SA, 2,3-DHBA and 2,5-DHBA standard curves were 0.999, 0.999 and 0.999, respectively.

ng SA in unknown = $(1.079 \times 10^{-5}) * (AUC)$

ng 2,3-DHBA in unknown = $(2.35 \times 10^{-6}) * (AUC)$

ng 2,5-DHBA in unknown = $(2.66 \times 10^{-6}) * (AUC)$

where AUC = area under curve for sample

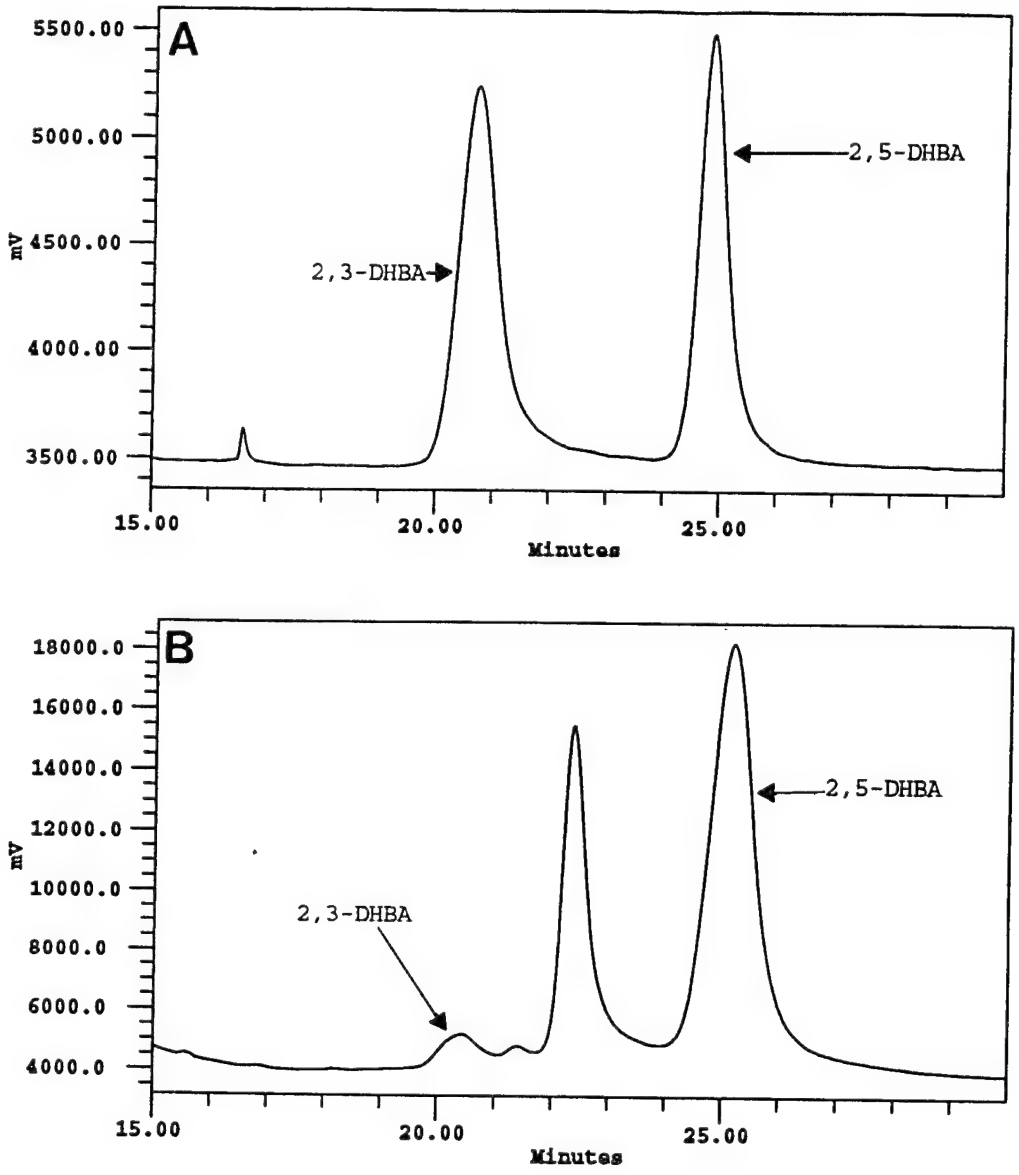


Figure 16A and 16B: Standard chromatographs for 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) standards are shown in panel A and a typical microsome sample chromatograph in panel B. The EC detector was set at 0.8 mV. The flow rate was set at 1.3 ml/min. using a 30 mM sodium citrate buffer (pH=4.65).

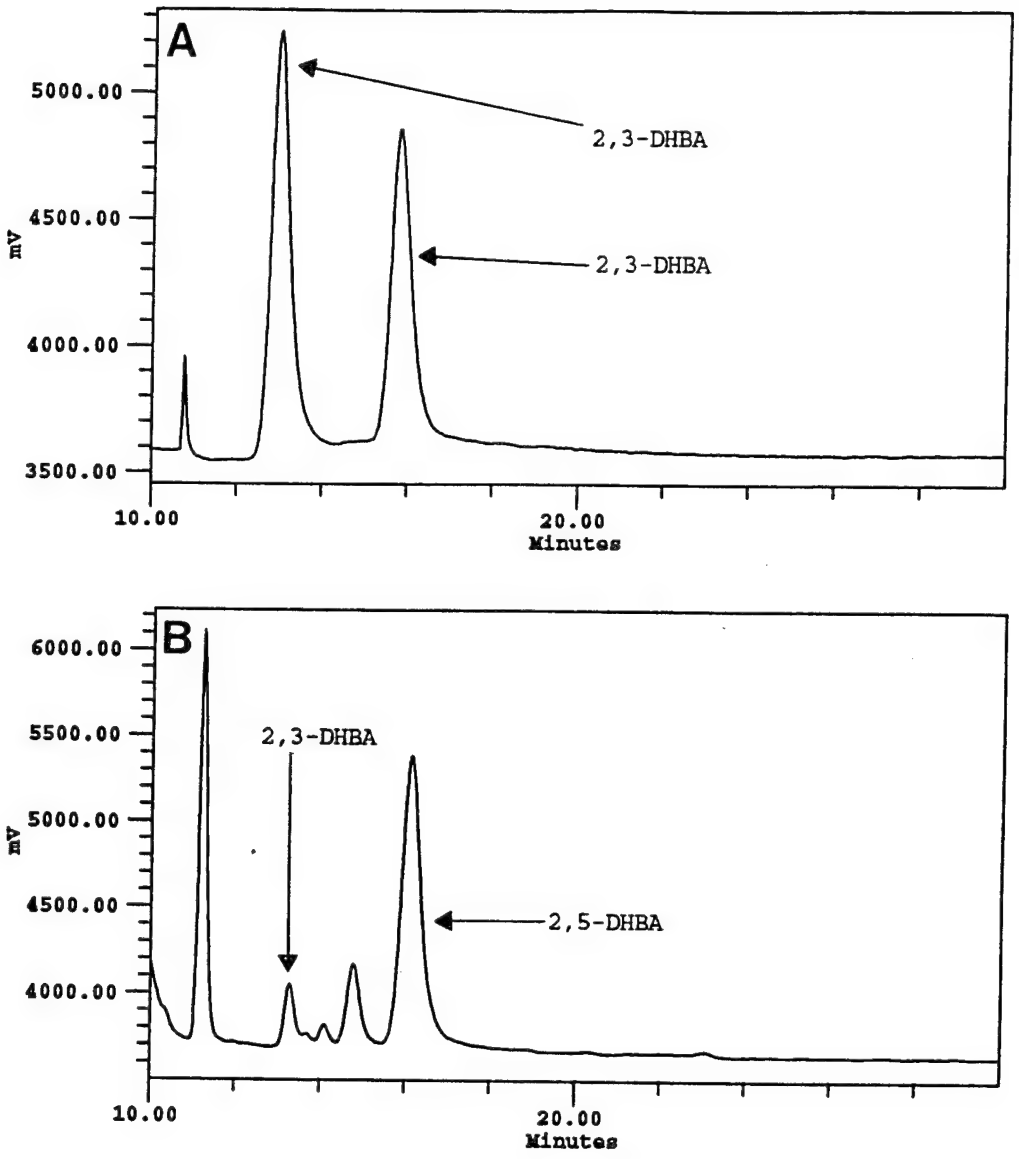


Figure 17A and 17B: Standard chromatographs for 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) standards are shown in panel A and a typical hepatocyte sample chromatograph in panel B. The EC detector was set at 0.8 mV. The flow rate was set at 1.3 ml/min. using a 30 mM sodium citrate buffer (pH=4.65).

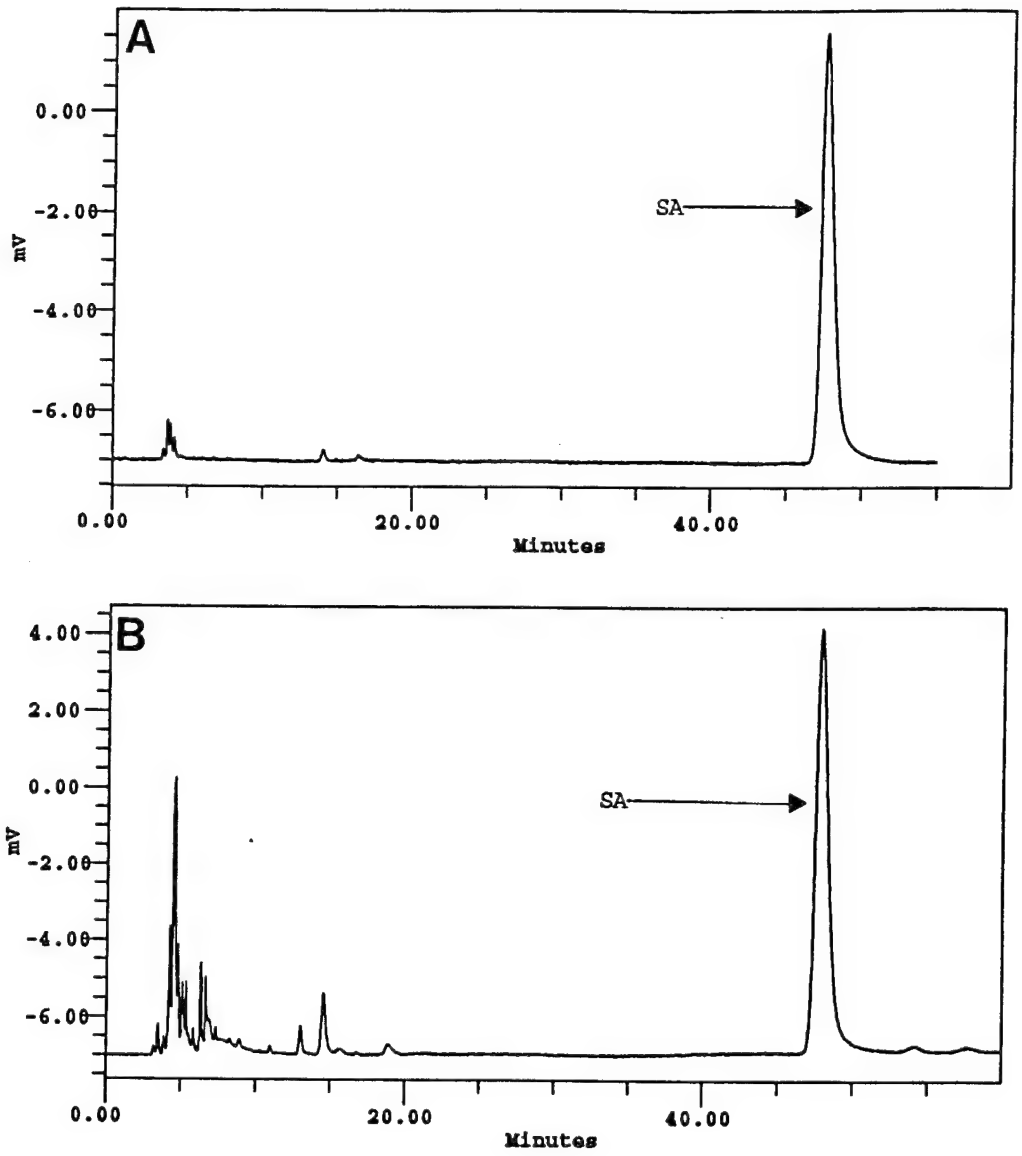


Figure 18A and 18B: A standard chromatograph for salicylic acid is shown in panel A and a typical sample chromatograph in panel B. The UV detector was set at 296 nm. The column was eluted with a mobile phase of 30 mM sodium citrate buffer (pH=4.65) with a flow rate of 1.3 ml/min.

F. ANIMAL SACRIFICE AND TISSUE COLLECTION

Rodents were euthanized by ethyl ether and exanguinated. One ml of blood was removed from the inferior vena cava and centrifuged at 6000 x g to isolate serum. At the same time, urine was extracted from the bladder using a syringe. The liver was removed, immediately frozen in liquid nitrogen and then stored at -80°C until analysis.

G. ANTIOXIDANT ENZYME ASSAYS

Livers were rinsed with ice-cold phosphate buffered saline (PBS) solution. The tissue was then placed in a solution of 0.32 M sucrose and 0.1 mM EDTA (1:4 weight:volume) and homogenized. A sample of the 20% tissue homogenate was used to measure protein content via Bio Rad Protein Assay Kit as previously described. Finally, the homogenate was stored in 1 ml aliquots at -80°C until ready for use.

1. Superoxide Dimutase

The method of Marklund and Marklund (1974) based on the inhibition of the auto-oxidation of pyrogallol was used to assay for SOD activity. One unit of enzyme activity equals amount of enzyme which inhibits auto oxidation of pyrogallol by 50%. The maximum inhibition obtainable is 97.5%. A frozen aliquot of homogenized tissue was thawed and diluted 1:8 with 0.32 M sucrose solution (25 mg wet tissue/ml sucrose solution). The homogenate

was next sonicated using three 12 second bursts with 15 second cooling periods on ice between burst. The resulting mixture was centrifuged at 20,000 x g for 20 minutes. The supernatant was removed to a clean eppendorf and stored at -80°C until ready for use.

The frozen supernatant was gently thawed in an ice bath. Two stock solutions were then prepared. The first solution consisted of 300 µl supernatant and 1 ml of 0.32 M sucrose (5.77mg tissue/ml) and the second solution consisted of 200 µl and 300 µl 0.32 M sucrose (10 mg tissue/ml). The assay was run on 200 µl aliquots of above dilutions (1.174 mg tissue and 2 mg tissue respectively). 750 µl of Tris-Cacodylic Buffer (50 mM Tris HCl, 50 mM Cacodylic acid , 1 mM Diethylenetriamine pentaacetic Acid (DTPA) adjust pH = 8.2), 250 µl of 2 mM Pyrogallol and 200 µl of sample were added to each test tube. The samples were immediately placed in the spectrophotometer and blanked. Absorbance was recorded for 5 minute. Rate of autoxidation equals increase in absorbance at 420 nm. 1 unit of enzyme = 50% inhibition of Pyrogallol oxidation.

2. Glutathione Peroxidase (GSH-Px)

One ml of 20% (200mg wet wt/ml) frozen homogenate was thawed and centrifuged at 105,000 x g for 60 minutes at 4°C. The supernatant was removed to a clean eppendorf and stored at -80°C until ready for use.

GSH-Px was measured by the method of Lawrence and Burk (1976)

based on the coupled reaction of oxidized glutathione (GSSG) formation by GSH-Px and reduction of GSSG by NADPH (Figure 19). In brief, sample was thawed and diluted 1:100 with PBS (136 mM NaCl, 2.68 mM KCl, 9.6 mM Na_2HPO_4, 1.47mM KH_2PO_4 pH=7.6). 800 µl of reaction mixture (1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH and 1 mM GSH in PBS buffer), 100 µl (10 units) of glutathione reductase and 100 µl of diluted enzyme sample were added to a 2 ml test tube. The mixture was vortexed and sat at room temperature for five minutes. The reaction was initiated by adding 100 µl of a 2.5 mM hydrogen peroxide solution. The reaction mixture was again vortexed and absorbance was recored for 5 minutes at 340 nm. A blank reaction was run by replacing enzyme sample with 100 µl dH_2O. The value of the blank was then substracted from each sample reading. Results were reported as µmoles NADPH oxidized per minute. Molar absorptivity for NADPH at 340 nm is 6.22×10^{-6}.

3. Glutathione S-Transferase (GST)

A solution of 50 mg tissue (wet weight) per ml of 0.32 M sucrose solution was centrifuged at 9000 x g for 15 minutes. The supernatant was transferred to a fresh tube and 1/10 volume of 0.1 M calcium chloride in 0.25 M sucrose was added. The solution was then centrifuged at 37,000 x g for 15 minutes. The supernatant was removed to a clean eppendorf and stored at -80°C until ready for use.

GST activity was determined by the method of Jacoby (1978) as modified by Benson (1978) based on the increase in absorbance at 340 nm for the catalyzed reaction of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) (Figure 19). In brief, 850 µl of a sodium phosphate buffer (0.1 M sodium phosphate and 1 mM EDTA buffer, pH = 6.5), 50 µl of a 20 mM GSH and 50 µl of a 20 mM CDNB in 95% ethanol were added to a 1 ml UV cuvette (all solutions preheated to 30°C). Next, 50 µl of isolated enzyme sample were added to reaction mixture and vortexed. Absorbance was measured at 340 nm. Correction for spontaneous reaction was made by measuring and subtracting the rate in absence of enzyme. A unit of enzyme activity = amount of enzyme that catalyzes the formation of 1 µmole of S-2,4-dinitrophenyl-glutathione per minute at 30°C using 1 mM of GSH and CDNB. The extinction coefficient at 340 nm is 10 $mM^{-1}cm^{-1}$(Yalcin et al., 1988).

4. Catalase

Five µl ethanol (0.17 M final concentration) were added to 500 µl of catalase supernatant and the solution was left to sit on ice for 30 minutes. Ethanol was added to increase the observable catalase activity by decomposing Complex II which is an inactive complex of catalase with hydrogen peroxide. Then, 55 µl of a 10% Triton X-100 solution were added. This improves activation of catalase during tissue homogenization. The mixture was vortexed 5 seconds and a 560 µl aliquot was removed. This was diluted to a final volume of 1ml by adding 440 µl of isotonic PBS yielding a final concentration of 100 mg wet tissue wt/ml of PBS. The assay was run on a 1mg wet tissue wt/ml with isotonic PBS which requires an additional 1:100 dilution. Catalase activity was then analyzed according to the method of Cohen et al., (1970) by monitoring the enzyme catalyzed decomposition of hydrogen peroxide using potassium permanganate and measuring absorbance at 480 nm.

The assay required four reactions to be run simultaneously. The first test tube contained 50 µl sample plus 50 µl of PBS, the second test tube contained 100 µl sample, the third test tube contained 100 µl water (blank) and the fourth test tube contained 100 µl 6 mM H_2O_2 solution (standard). One ml of cold 6 mM H_2O_2 solution was then added to each test tube and mixture was vortexed. The reaction mixture sat at room temperature for three minutes, then 200 µl 6 N H_2SO_4 were added to each test tube to stop

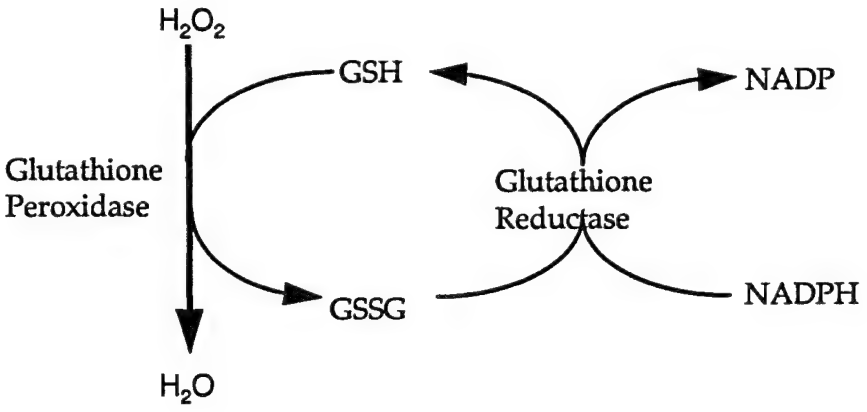
reaction. Finally, 1.4 ml 0.01 N $KMnO_4$ were next added to each test tube and mixture was vortexed. Absorbance was recorded at 480 nm within 30-60 seconds to avoid precipitation of reactants. One unit of enzyme activity equals k/(0.00693) (Aebi, 1974), where k = $\log(S_0/S_2)$ x (2.3/t), S_0 = absorbance of standard - absorbance of blank, S_2 = absorbance of standard - absorbance of sample and t = time interval (3 minutes in this case).

H. ASSAY FOR VITAMIN E

The method of Arnaud et al., (1991) was used for analysis of vitamin E (α-tocopherol). In brief, tissue was placed in deionized water in the ratio of 1 mg tissue to 10 µl of cold deionized water. The tissue was homogenized using a Potter-Elvehjem Tissue Grinder with PTFE pestle (Fisher Sci. Co.) for 10 seconds. 300 µl of tissue homogenate were removed and placed in a 2 ml conical tube with screw top. Next, 300 µl of a 0.1 M SDS solution were added to the homogenate and vortexed gently for 15 seconds. Then, 600 µl of cold ethyl alcohol was added and again vortexed for 30 seconds. To this mixture was added 600 µl of hexane and mixture was mixed by shaking for 5 minutes. The mixture was centrifuge at 3000 x g for 5 minutes and 400 µl (20 mg tissue) of top hexane layer was removed to a new 2 ml conical tube. Hexane was evaporated off under nitrogen gas and residue was redissolved in 200 µl of the 20% dichloromethane: 70% acetonitrile: 10% methanol mobile phase (10 µl of mobile phase contains vitamin from 1 mg tissue).

If cell cultures were used, culture media was removed and cells were rinsed twice with phosphate buffer saline solution (pH=7.0). Buffer was removed and 0.5 mls of deionized water were added. Cells were scraped off culture dish and homogenize using a Potter-Elvehjem Tissue Grinder with PTFE pestle (Fisher Sci. Co.) for 10 seconds. 7 to 10 million cells were used for analysis. Homogenate was assayed for protein content and 300 ul of homogenate were removed for vitamin E assay. Next, the procedure was followed as listed above for tissue vitamin E extraction.

A.



B.

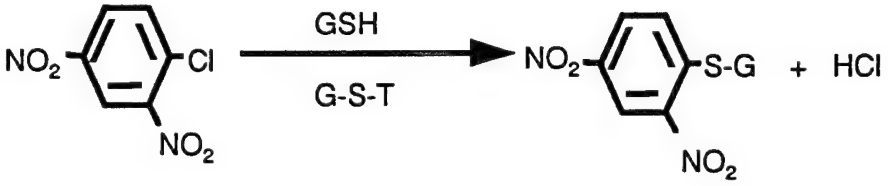


Figure 19: Reaction Systems used to Assay Glutathione Peroxidase and Glutathione S-Transferase Activity

A. Coupled reaction of glutathione reductase with glutathione peroxidase. GSH-Px is measured by monitoring decrease in absorbance of NADPH at 340nm over a 5 minute interval.

B. Colorometric reaction of 1-chloro-2,4-dinitrobenzene and glutathione to form S-2,4-dinitrophenylglutathione. Rate of formation is monitored at 340 nm.

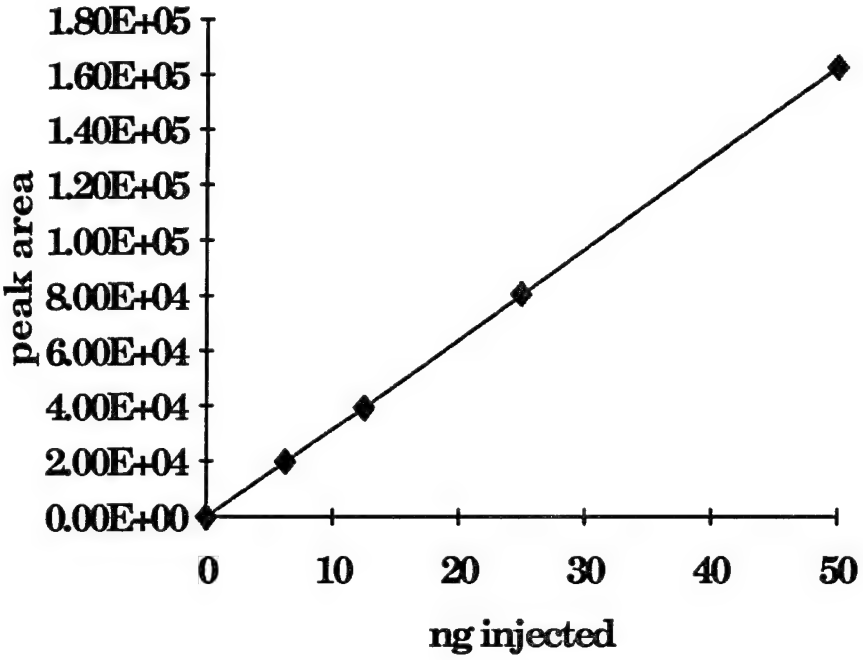


Figure 20: Calibration curve for Vit E. Standards were run at the start of each experiment and at the conclusion. r^2 value for standard curve equals 0.999 ng Vit E in unknown = 3.07×10^{-4} *(AUC).

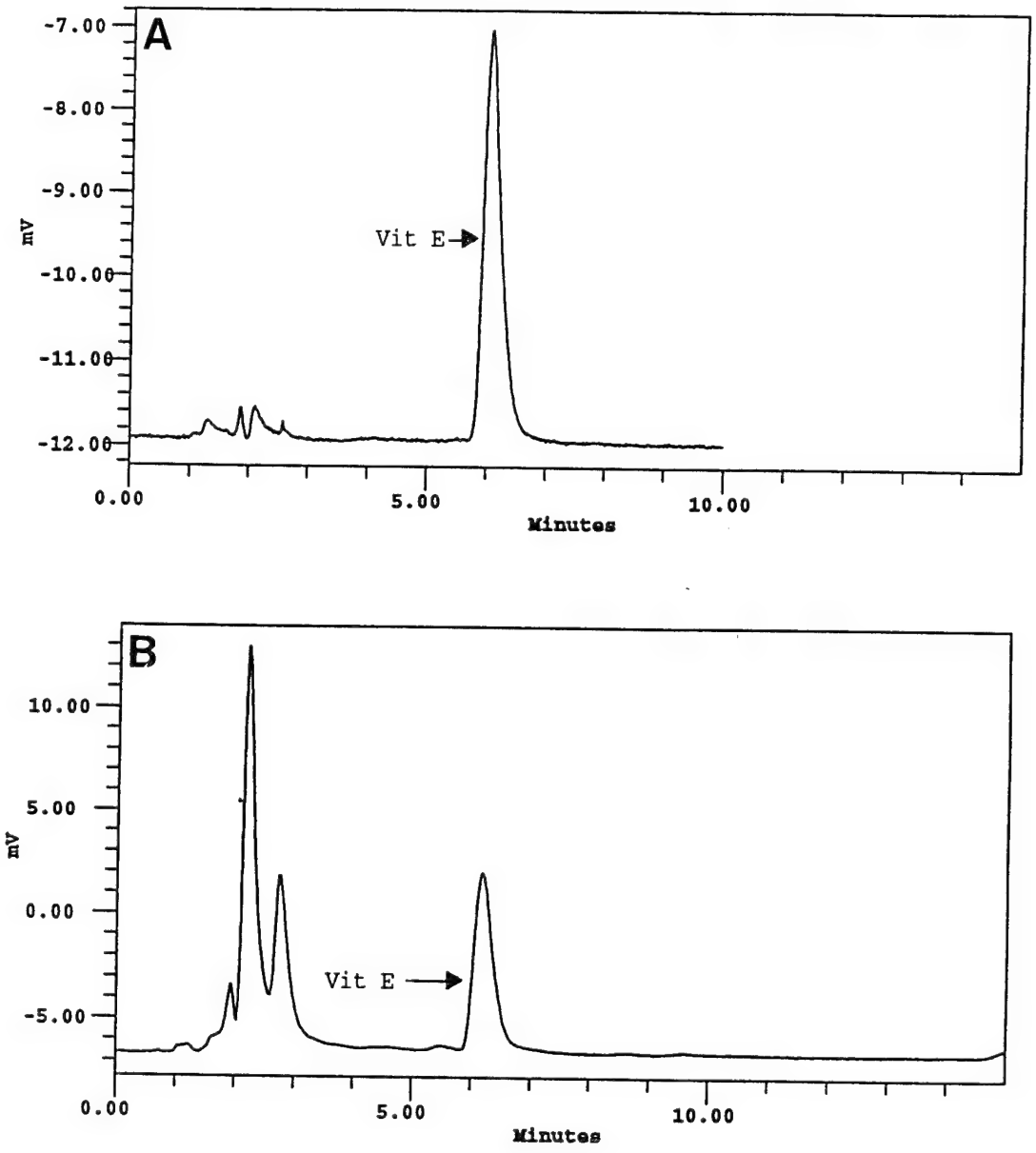


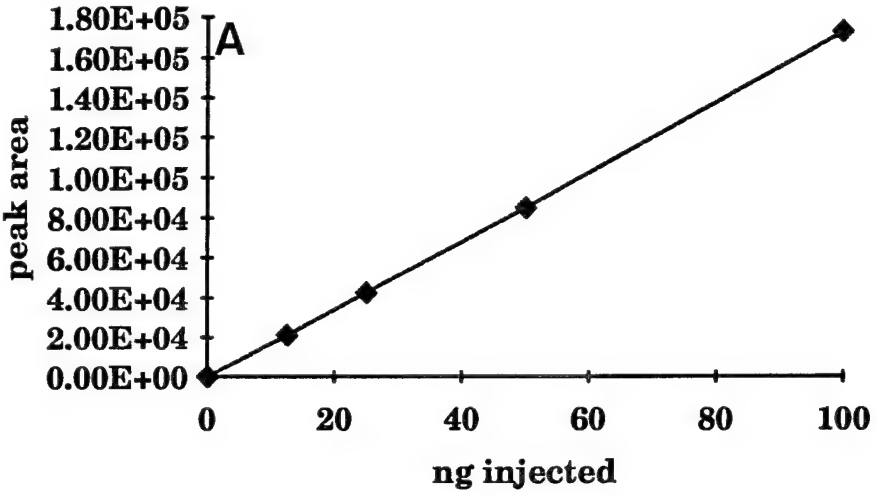
Figure 21A and 21B: A typical chromatograph for Vit E is shown in panel A and a typical sample chromatograph is shown in panel B. The UV detector was set at 291 nm. The flow rate was set at 1.0 ml/min. using a 20:70:10 dichloromethane:acetonitrile:methanol mobile phase.

The HPLC system consisted of a Waters 600E pump with a Waters 700 Satellite WISP autoinjector controlled by a Waters Millennium 2010 software package using an IBM 486 computer. The chromatography system consisted of one Beckman Ultrasphere ODS C18 column, 5 μ, 150x4.6 mm column. The column was eluted with a 20% dichloromethane: 70% acetonitrile: 10% methanol mobile phase at a flow rate of 1.0 ml/min. Vit E was detected on a Waters 484 Tunable Absorbance Detector was set at 291 nm. Vit E standard elutes at ~6 minutes. 10 μl of unknowns were injected for analysis. The run time for each sample was ~15 minutes and samples were kept on ice until injected. A calibration for Vit E is shown in Figure 20. A typical chromatograph is shown in Figure 21.

I. ASSAYS FOR GLUTATHIONE, VITAMIN C AND URIC ACID

Tissue GSH, Vit C and UA were simultaneously analyzed by a HPLC-EC (Quiroga et al., (1991); Washko et al., (1989) and Harvey et al., (1989)). Briefly, 200 mg of tissue or 3.5 million cells were homogenized in 1 ml of cold 0.1 M perchloric acid with 2 mM EDTA in a Potter-Elvehjem Tissue Grinder with PTFE pestle (Fisher Sci. Co.) for 10 seconds. The homogenate was kept on ice for 10-15 minutes and then centrifuged at 5,000 x g (0-4°C) for 5 minutes in a Beckman J2-MI centrifuge (Beckman Co.). The supernant was diluted 10 times with HPLC mobile and immediately injected into HPLC for analysis. Samples were kept at 0-4 °C in whole period from tissue treatment

to sample analysis. The HPLC system consisted of a Waters 600E pump with a Waters 700 Satellite WISP autoinjector controlled by a Millennium 2010 software in an IBM computer (Millipore, Waters Chromatography). Two 8 x 100 mm Waters Nova-Pak C18 columns, 4 μ, in a Radial-Pak cartridge with a extension kit protected by a Nova-Pak C18 Guard-Pak Insert were eluted with 1.5% aqueous acetonitrile containing 50 mM ammonium phosphate (pH3.4) at 1 ml/min flow rate. A CC-5/LC-4C Ameperometer Detector from BAS System(West Lafayette, IN) was set at +1.0 V potential and 0.1 filter. Under these condition, detectable limits were 0.1 ng for Vit C and uric acid and 50 ng for GSH. Standards were prepared in a 50mM perchloric acid solution. Standard curves for UA and Vit C are shown in figure 22 and for GSH in figure 23. Typical chromatographs for UA, Vit C and GSH are shown in figures 24, 25 and 26, respectively.



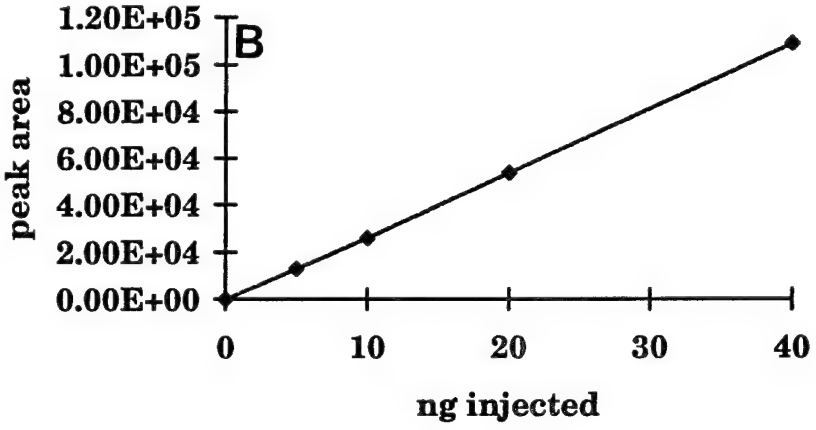


Figure 22A and 22B: Calibration curves for UA and Vit C are shown in panel A and B respectively. Standards were run at the start of each experiment and at the conclusion. r^2 values for standard curves were 0.999 for UA, 0.999 for Vit C.
ng UA in unknown = 3.70×10^{-6}*(AUC)
ng VC in unknown = 2.079×10^{-6}*(AUC)

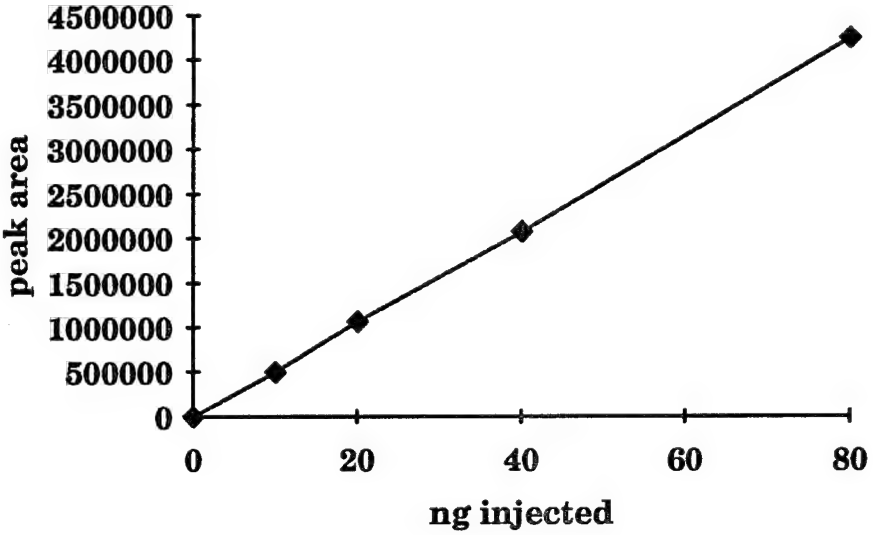


Figure 23: Calibration curve for glutathione (GSH). Standards were run at the start of each experiment and at the conclusion. r^2 value for standard curve was 0.999 ng GSH in unknown = 6.14×10^{-8} *(AUC).

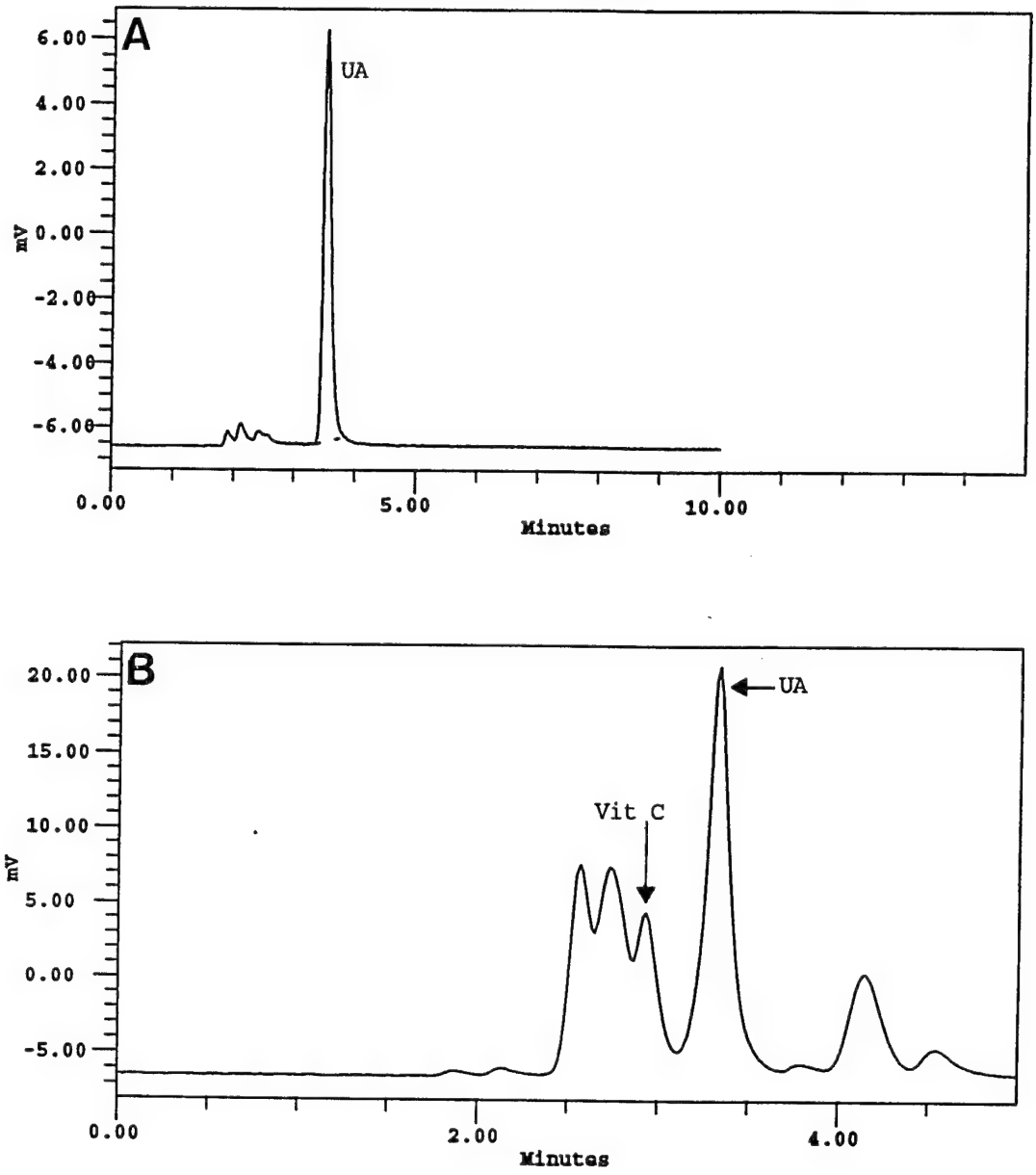


Figure 24A and 24B: A standard chromatograph for UA is shown in panel A and a typical sample chromatograph in panel B. The UV detector was set at 260 nm and the flow rate was set at 1.0 ml/min. using a 50 mM ammonium phosphate (pH=3.4) containing 1.5% aqueous acetonitrile.

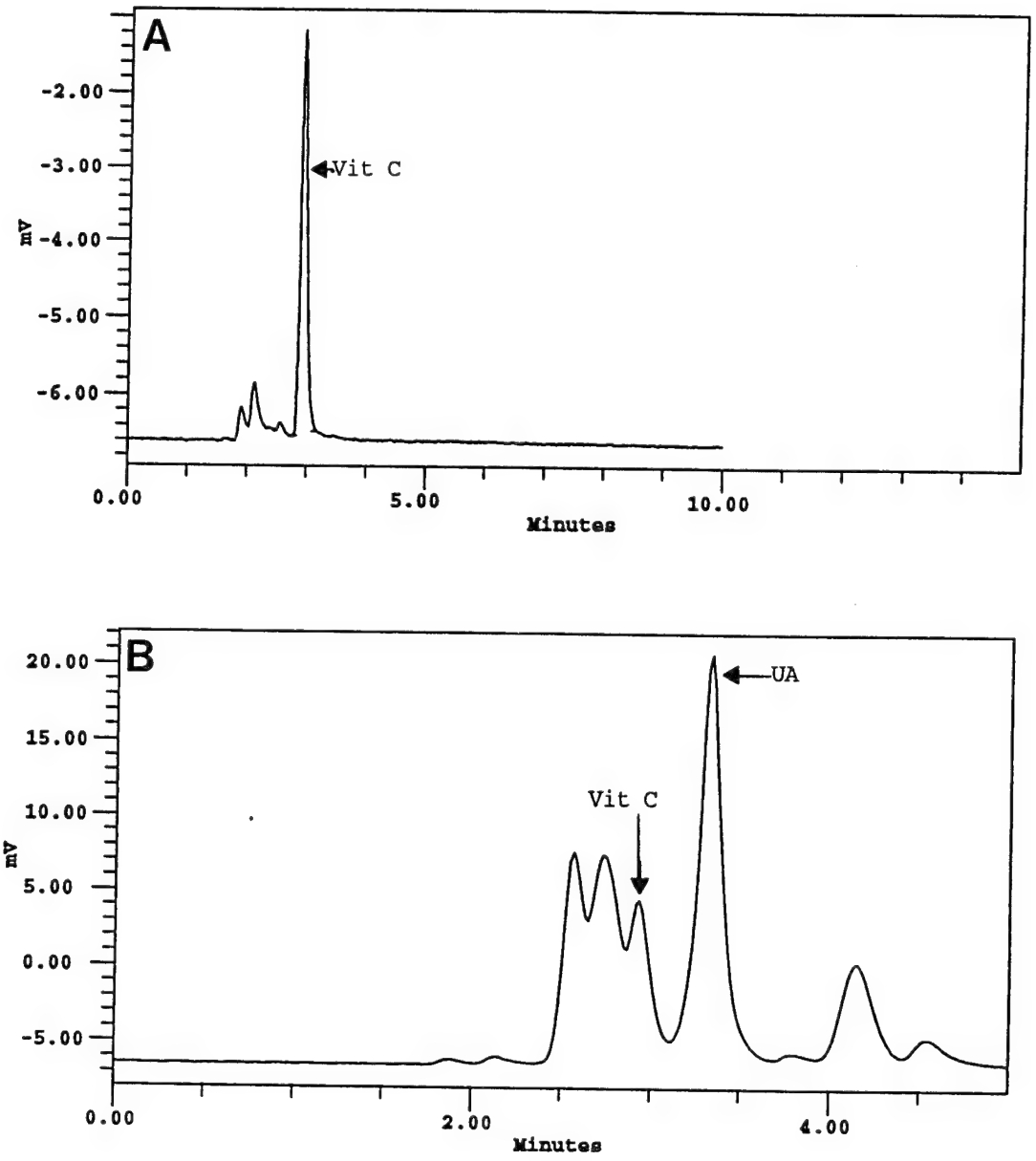


Figure 25A and 25B: A standard chromatograph for Vit C is shown in panel A and a typical sample chromatograph in panel B. The UV detector was set at 260 nm and the flow rate was set at 1.0 ml/min. using a 50 mM ammonium phosphate (pH=3.4) containing 1.5% aqueous acetonitrile.

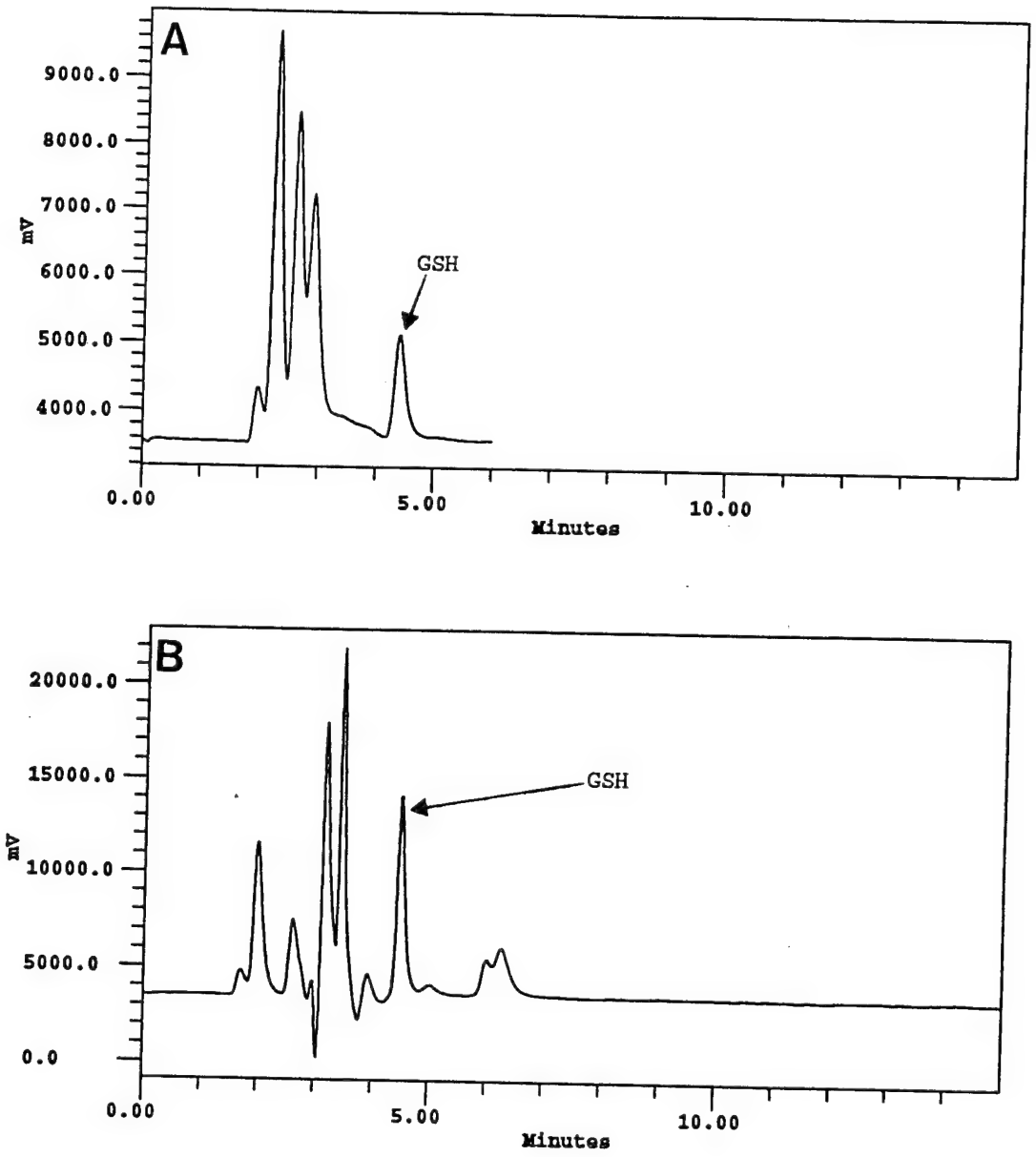


Figure 26A and 26B: A Standard chromatograph for GSH is shown in panel A and a typical sample chromatograph in panel B. The EC detector was set at 1.0 mV. The flow rate was set at 1.0 ml/min. using a 50 mM ammonium phosphate (pH=3.4) containing 1.5% aqueous acetonitrile.

J. MALONDIALDEHYDE ASSAY (THIOBARBITURIC ACID METHOD)

The thiobarbituric acid reactive species (TBARS) assay followed the method of Uchiyama and Mihara (1978) (Figure 27). In brief, one million cells were required per assay. Cells were removed from incubator and washed two times with cold phosphate buffer saline solution (PBS) (136 mM Sodium Choride, 2.68 mM Potassium Choride, 9.6 mM Sodium Phosphate Dibasic, 1.47 mM Potassium Phosphate Monobasic pH=7.6). PBS solution was removed and 500 ul of citrate buffer (15 mM NaCl, 50 mM Trisodium Citrate with pH = 7.0) were added to each dish. Cells were scraped off with a rubber policeman and homogenized on ice using polytron homogenizer. 20 ul of homogenate were removed to quantify protein content. 200 ul of homogenate were removed and added to 200 ul of a 38% solution (v/v) of trichloroacetic acid to precipitate protein (final pH = 2.5) (verified pH by using pHydrion paper 1 to 2.5 and 3.0 to 5.5). 1 ml of TBAR reagent (2 parts of 1% Thiobarbituric acid to 1 part 0.5 M sodium citrate; pH to 7.0) was added to each sample and heated at 95°C for 30 minutes. pH should remain at 2.5. Samples were cooled to room temperature and centrifuged at 12,000 x g for 10 minutes. Absorbance was measured at 530 nm. Results were expressed as nmoles/mg protein. A standard curve was run each day of analysis. A 20 mM stock solution of 1,1,3,3-tetramethoxypropane was prepared which quickly hydrolyzes into malondialdehyde. 0.5 to 10 μMole standards were prepared for calibration curve using the following table:

a. 0.5 µMole	25 ul stock B	200 µl 38% TCA	775 µl Reagent A
b. 1 µMole	50 µl stock B	200 ul 38% TCA	750 µl Reagent A
c. 2 µMole	125 µl stock B	200 µl 38% TCA	675 µl Reagent A
d. 4 µMole	200 µl stock B	200 µl 38% TCA	600 µl Reagent A
e. 8 µMole	400 µl stock B	200 µl 38% TCA	400 µl Reagent A

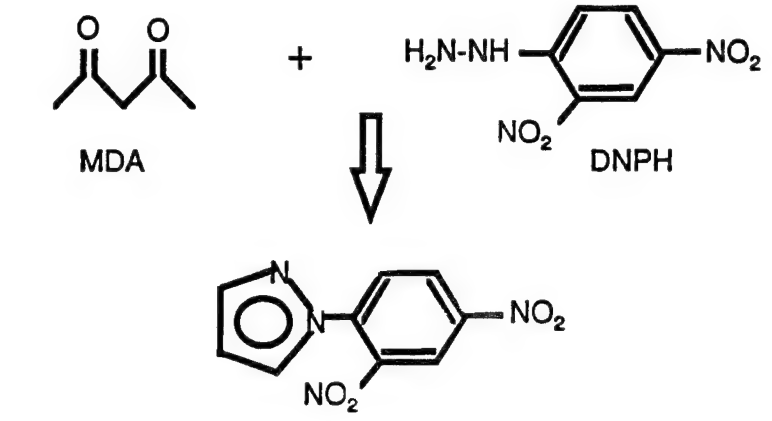
Standard mixtures were heated at 95°C for 30 minutes. pH should remain at 2.5. Standards were cooled to room temperature and centrifuged at 12,000 x g for 10 minutes. Absorbance was then recorded.

K. MALONDIALDEHYDE ASSAY (HYDRAZINE DERIVATIVE METHOD)

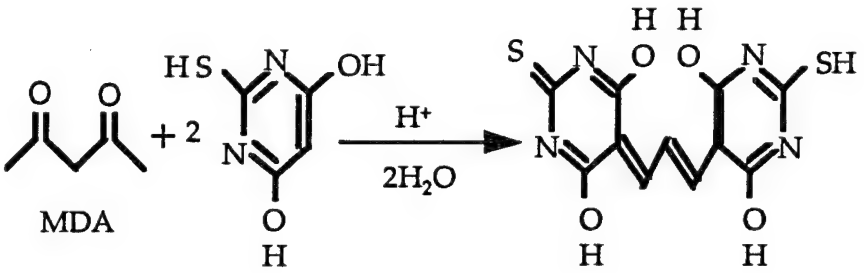
The method of Bagchi et al., (1993) was used for analysis of urinary and tissue MDA (Figure 27). Briefly, 100 mg of tissue was homogenized in 900 ul of cold 0.1 M perchloric acid with 2 mM EDTA in a Potter-Elvehjem Tissue Grinder with PTFE pestle (Fisher Sci. Co.) for 10 seconds. The homogenate was kept on ice for 10-15 minutes, and then centrifuged at 5,000 x g (0-4°C) for 5 minutes in a Beckman J2-MI centrifuge (Beckman Co.). 500 ul of the supernant (or 50 µl of urine and 450 µl deionized water) were removed and placed in a 1.5 ml eppendorf tube. To this clear liquid was added 50 ul of 15.65 mM 2,4-dinitrophenylhydrazine (DNPH) solution prepared in 2 N HCL. The solution was left to sit at room temperature for 60 minutes. Then, 800 µl of pentane were added and the mixture was shaken

rigorously for 10 minutes, centrifuged at 5,000 x g and 400 ul of the top pentane layer were removed to a fresh 2 ml conical tube with screw top. Pentane was evaporated off under nitrogen gas. Residue was redissolved in 250 µl of mobile phase (1 mg tissue per 5 µl mobile phase).

The HPLC system consisted of a Waters 600E pump with a Waters 700 Satellite WISP autoinjector controlled by a Waters Millennium 2010 software package using an IBM 486 computer. The chromatography system consisted of one Beckman Ultrasphere ODS C18 column, 5 µ, 150 x 4.6 mm column. The column was eluted with a 49% aqueous acetonitrile and 51% water mobile phase at a flow rate of 1.0 ml/min. The malondialdehyde-hydrazine adduct was detected on a Waters 484 Tunable Absorbance Detector set at 307 nm. The standard eluted at ~5.2 minutes. 100 µl of unknowns were injected for analysis. The run time was ~ 25 minutes per sample and samples were kept on ice until injected. A calibration curve is shown in figure 28. A typical urine chromatograph is shown in figure 29 and a typical liver chromatograph is shown in figure 30. Results were expressed as nmoles MDA per gram tissue or nmoles MDA per mg creatinine. Creatinine was measure using a creatinine detection kit (SIGMA procedure No. 555) based on reaction of creatinine with acidic picrate. A COBAS MiraS spectrophotometer calibrated with standard creatinine solutions (SIGMA 925-3 and 925-15) was used to measure difference in color intensity at 500 nm before and after acidification of creatinine-picrate complex.



A: MDA reaction with DNPH



B. MDA reaction with Thiobarbituric Acid

Figure 27: Analysis of Malondialdehyde by A) The HPLC Dinitrophenolhydrazine (DNPH) method or B) The UV Spectrometer Thiobarbituric Assay Technique

Reaction of Malondialdehyde (MDA) with 2,4-dinitrophenylhydrazine (DNPH) to form a pyrazole derivative for analysis by HPLC is shown in panel A. The Thiobarbituric Acid adduct with MDA is shown in panel B. The Thiobarbituric Acid Reactive Species (TBARS) is measured on UV at 535 nm.

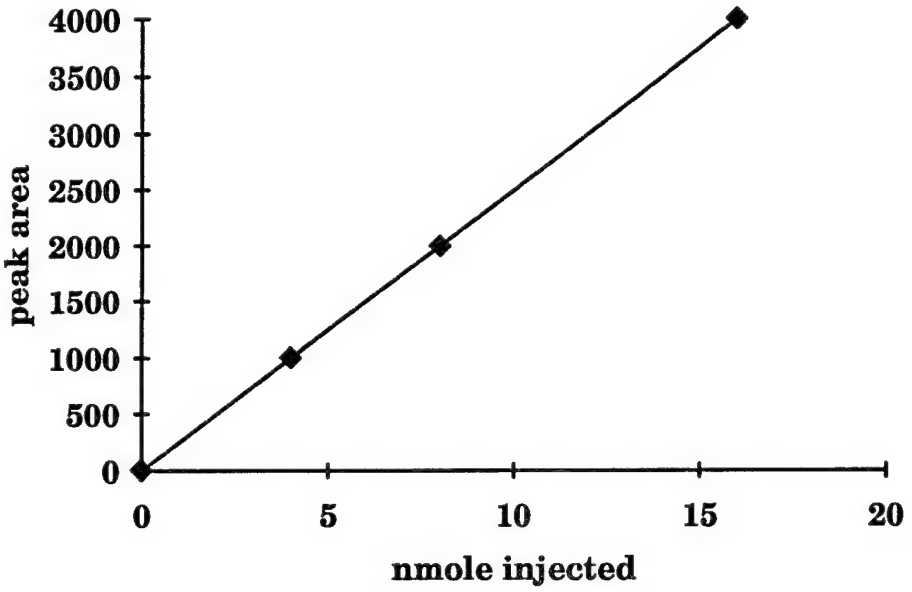


Figure 29: Calibration curve for malondialdehyde-hydrazine adduct. Standards were run at the start of each experiment and at the conclusion. r^2 value was 0.996. nmole MDA in unknown = 8.69×10^{-5} *(AUC).

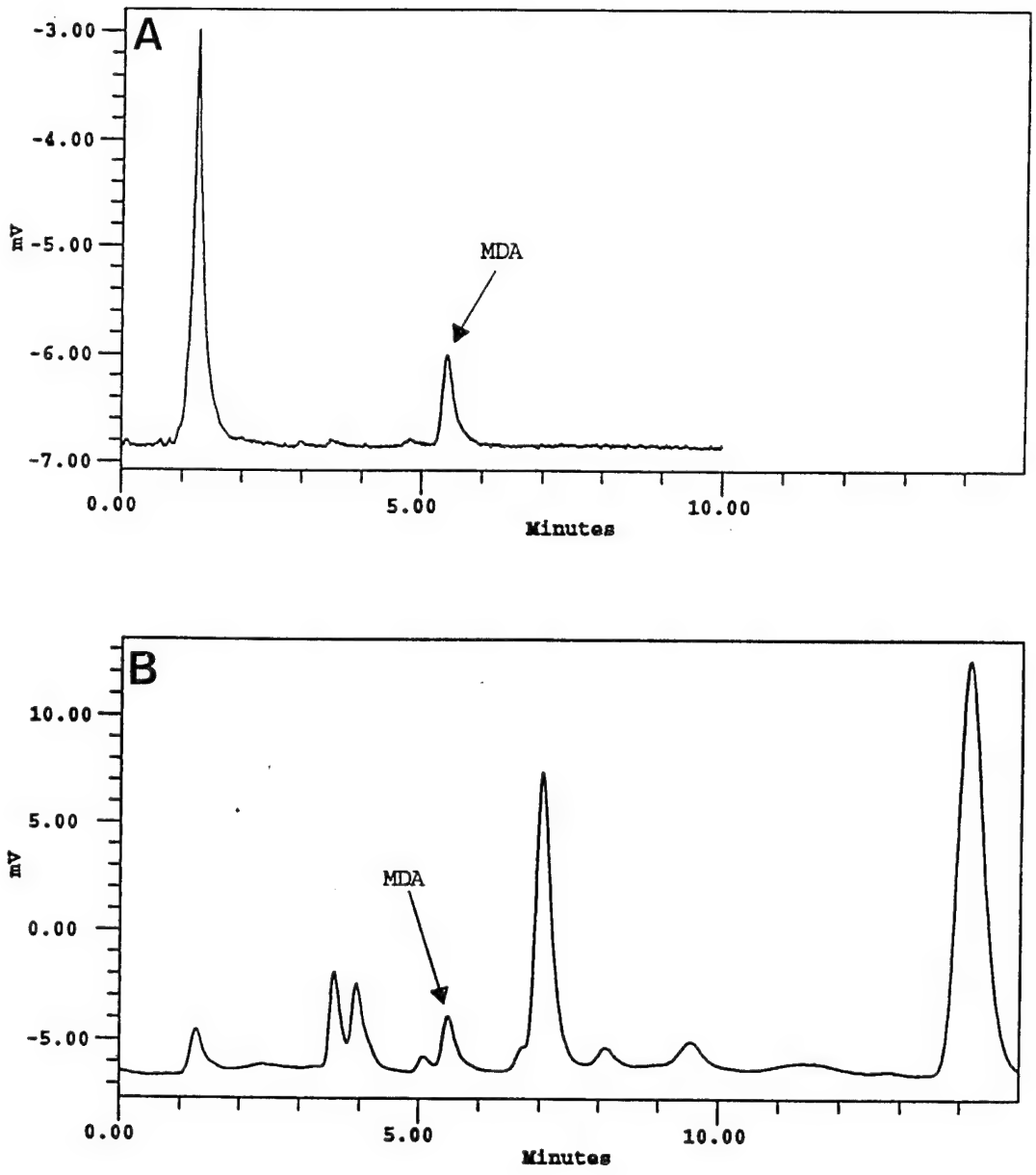


Figure 30A and 30B: A chromatograph for malondialdehyde-hydrazine adduct is shown in panel A and a typical tissue sample chromatograph is shown in panel B. The UV detector was set at 307 nm. The flow rate was set at 1.0 ml/min. using a 49:51 acetonitrile:water mobile phase.

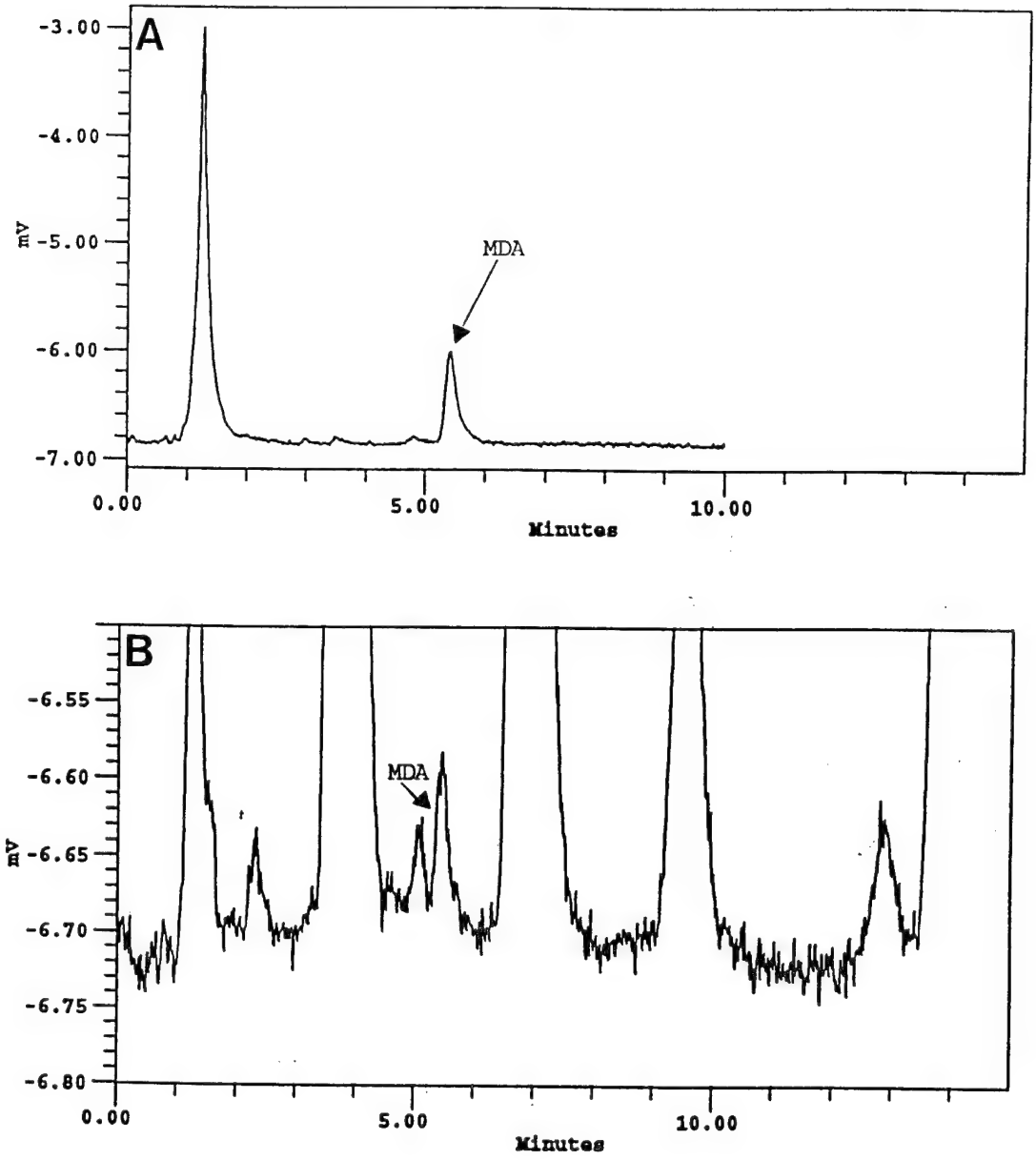


Figure 31A and 31B: A chromatograph for malondialdehyde-hydrazine adduct is shown in panel A and a typical urine sample chromatograph is shown in panel B. The UV detector was set at 307 nm. The flow rate was set at 1.0 ml/min. using a 49:51 acetonitrile:water mobile phase.

L. ASSAY FOR HEPATIC OH8DG

Cell DNA was isolated by a modified procedure of Marmur's (1961) for HPLC analysis of oh8dG as described by Chung et al., (1992). At least one million cells (*in vitro*) or 200 mg tissue (*in vivo*) were required for analysis. The procedure was divided into three parts: (1) DNA isolation, (2) DNA digestion and (3) HPLC analysis.

1. DNA Isolation

For *in vitro* work, the media was removed from a 100 mm culture dish containing one million hepatocytes and the cells were washed twice with 1 ml 15 mM NaCL-50 mM Trisodium Citrate (pH=7.0). After removing the buffer, 0.5 ml of 15 mM NaCL-50 mM and Trisodium Citrate (pH=7.0) were added into each dish for cell collection. Collected cells were transferred to a 1.5 ml eppendorf tube and centrifuged (6,000 x g). The supernatant was discarded and 0.5 ml of lysis buffer (10 mM Tris-1 mM EDTA-10%SDS, pH=7.0) was added to pellet. To this mixture was added 5 units of proteinase K and the mixture was placed in a water bath at 37°C for 20 minutes.

For *in vivo* work, 1 ml of 15 mM NaCL and 50 mM Trisodium Citrate (pH=7.0) was added to 200-400 mg of tissue. The tissue was homogenized in a stainless steel tissue homogenizer, transferred to a 1.5 ml eppendorf tube and centrifuged (6,000 x g). The supernatant was discarded and 0.5 ml of lysis buffer (10 mM Tris-1 mM EDTA-10%SDS, pH=7.0) were added and cells

were broken with pipette. To the mixture was added 20 units of proteinase K and the mixture was placed in a water bath at 37°C for 20 minutes.

From this point, the procedure proceeded identically for both *in vitro* and *in vivo* DNA analysis. After digestion with proteinase K, the mixture was extracted with 0.5 ml chloroform:iso amylalcohol (24:1) by shaking 5 minutes by hand. The mixture was centrifuged at 12,000 x g for 10 minutes and the upper layer transferred to a new 1.5 ml eppendorf tube. Again, 0.5 mls of chloroform:iso-amyl alcohol (24:1) were added. The extraction was repeated and the supernatant (~0.5 ml) was placed in a fresh 1.5 ml eppendorf. Sixty ul of 2 M NaCl and 1 ml of cold ethyl alcohol was added to each sample, the mixture was rigorously shaken and centrifuged (12,000 x g) for 15 minutes. Liquid was decanted off completely and DNA was redissolved in 0.5 ml 10 mM Tris-HCL (pH=7.0). Five uints of RNase A were added to sample and mixtures were placed in water bath at 37°C for 10 minutes. Samples were removed from water bath and 5 units (10 units for tissue) of proteinase K were added. Solutions were vortex gently and placed in water bath at 37°C for an additional 30 minutes. As before the protein was extracted the with 0.5 mls chloroform:iso-amyl alcohol (24:1) by shaking 5 minutes by hand, centrifuged at 12,000 x g for 10 minutes and the upper layer transferred to a new 1.5 ml eppendorf tube. Again, 0.5 mls of chloroform:iso-amylalcohol (24:1) were added to each eppendorf and the extraction was repeated. Upper layer was tranferred to a clean 1.5 ml

eppendorf (~0.5 ml). 60 µl 2M NaCL and 1 ml of cold ethyl alcohol were added to solutions. Mixtures were rigorously shaken and centrifuged (12,000 x g) for 15 minutes. Liquid was completely decanted off and DNA samples were stored at -80°C until ready for use.

2. DNA digestion

DNA samples were removed from -80°C freezer and 200 µl 10 mM Tris HCl buffer were added to each sample. Mixtures were vortexed gently to help dissolve DNA. (The DNA may not completely dissolve at this point but should after being placed in water bath.) To each eppendorf tube, 10 µl 0.5 M sodium acetate buffer and 10 units nucease PI were added. Mixtures were incubated in a waterbath at 37°C for 60 minutes. After 60 minutes, solutions were removed from the water bath. 40 µl of 0.4 M Tris HCl buffer and 14 units of alkaline phosphatase were then added to each eppendorf. Solutions were again incubated in water bath at 37°C for 60 minutes. After 60 minutes, solutions were removed from the water bath and centrifuged at 12,000 x g for 10 minutes at 4°C. Supernatants were removed for HPLC analysis. If tissue was used for DNA isolation, it may be necessary to prefilter, the supernatant through a 0.22 micron microcentrifuge tube filter prior to HPLC analysis.

3. HPLC analysis

The HPLC system consisted of a Waters 600E pump with a Waters 700 Satellite WISP autoinjector controlled by a Waters Millennium 2010 software package using an IBM 486 computer. The chromatography system consisted of two Waters Nova-Pak C18 column, 4 μ, 8 x 100 mm in a Radial-Pak catridge guarded by a Nova-Pak C18 Guard-Pak Insert. The column was eluted with a 90% 12.5 mM sodium citrate, 25 mM sodium acetate and 10 mM acetic acid buffer with 10% methanol at a flow rate of 1.0 ml/min. Deoxyguanosine was detected on a Waters 484 Tunable Absorbance Detector was set at 260 nm. oh8dG was detected on a CC-5/LC-4C Ameperometer Detector from BAS System set at 0.1 μA range, +600 mV potential and 0.1 filter for oh8dG detection. The oh8dG standard eluted at ~16 minutes while the dGuo standard eluted at ~14 minutes. 150 μl for of unknowns were injected for analysis. The run time for each sample was ~ 22 minutes and samples were kept on ice until injected. Calibration curves for oh8dG and dGuo are shown in figure 31. A typical liver dGuo is shown in figure 32. Figure 33 shows a typical liver chromatograph for oh8dG.

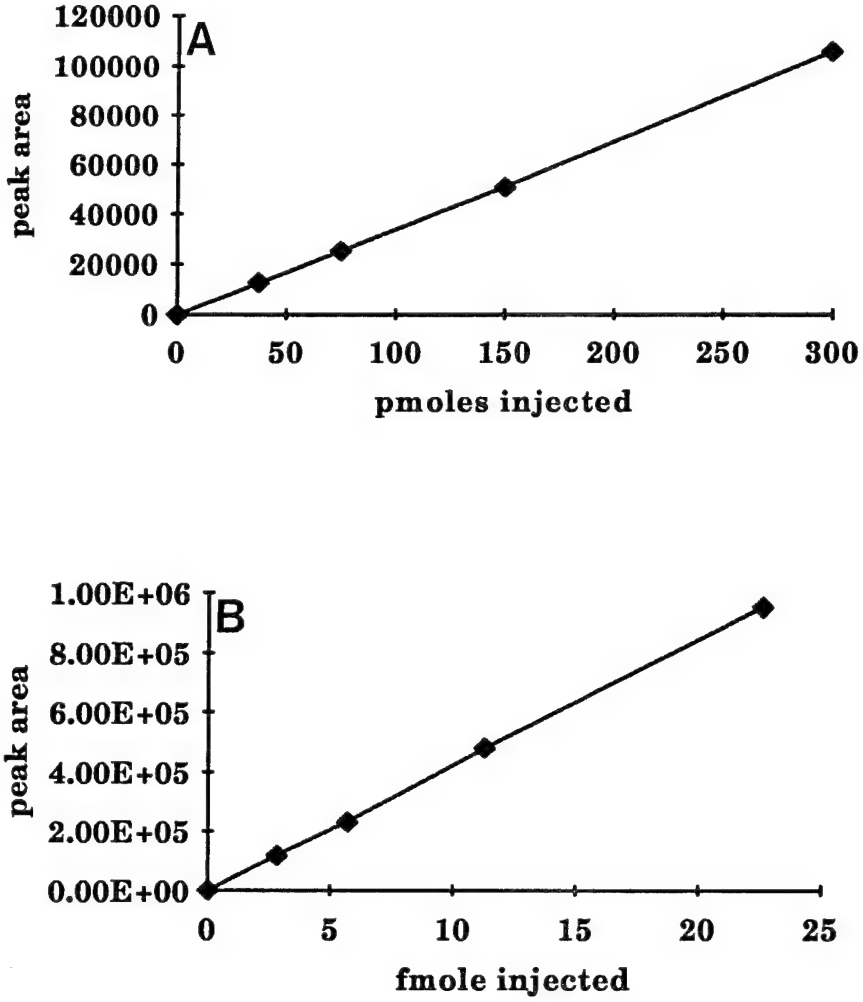


Figure 31A and 31B: Calibration curves for deoxyguanosine (dGuo) and 8-hydroxy-2'-deoxyguanosine (oh8dG) are shown in panels A and B respectively. Standards were run at the start of each experiment and at the conclusion. r^2 values were 0.999 for oh8dG and 0.999 for dGuo.
pmoles dGuo in unknown = 0.00282 * (AUC)
fmoles oh8dG in unknown = 2.375×10^{-5} * (AUC)

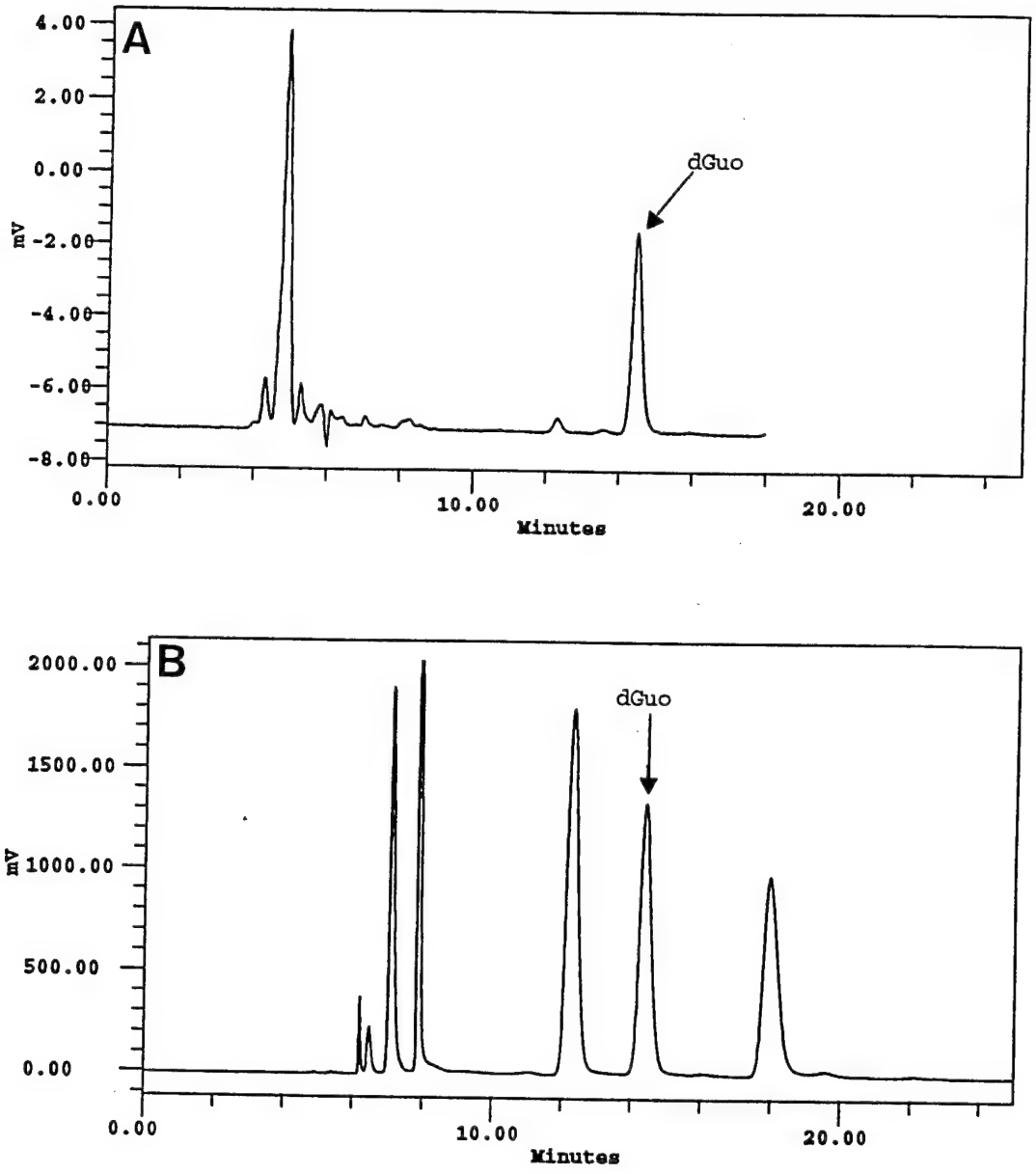


Figure 32A and 32B: A standard chromatograph for deoxyguanosine is shown in panel A and a typical sample chromatograph in panel B. The UV detector is set at 260 nm. The flow rate was set at 1.0ml/min. using a 90% 12.5 mM sodium citrate, 25 mM sodium acetate and 10 mM acetic acid buffer with 10% methanol.

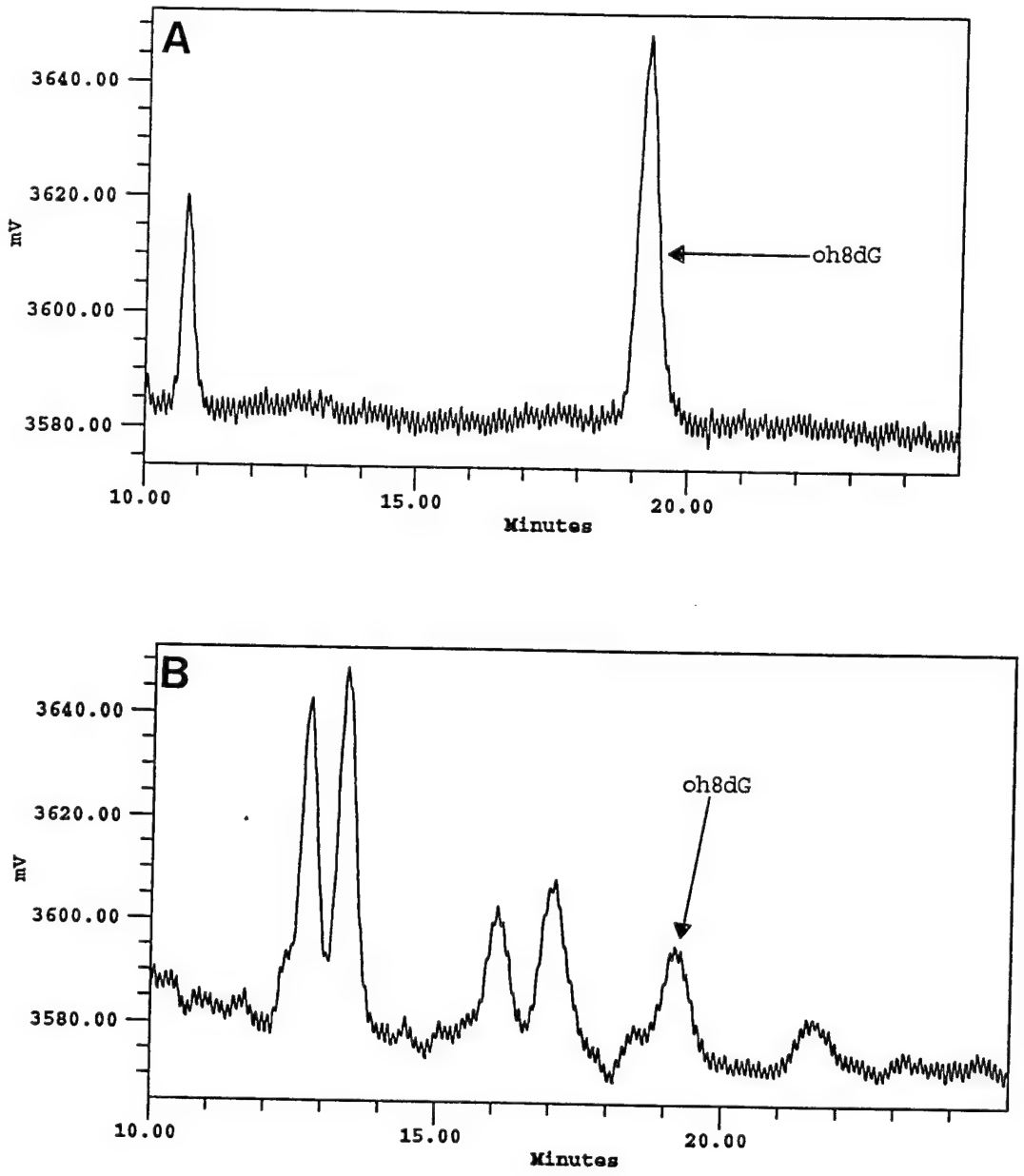


Figure 33A and 33B: A standard chromatograph for 8-hydroxy-2'-deoxyquanosine (oh8dG) standard is shown in panel A and a typical liver sample chromatograph in panel B. The EC detector was set at 0.6 mV. The flow rate was set at 1.0 ml/min. using a 90% 12.5 mM sodium citrate, 25 mM sodium acetate and 10 mM acetic acid buffer with 10% methanol.

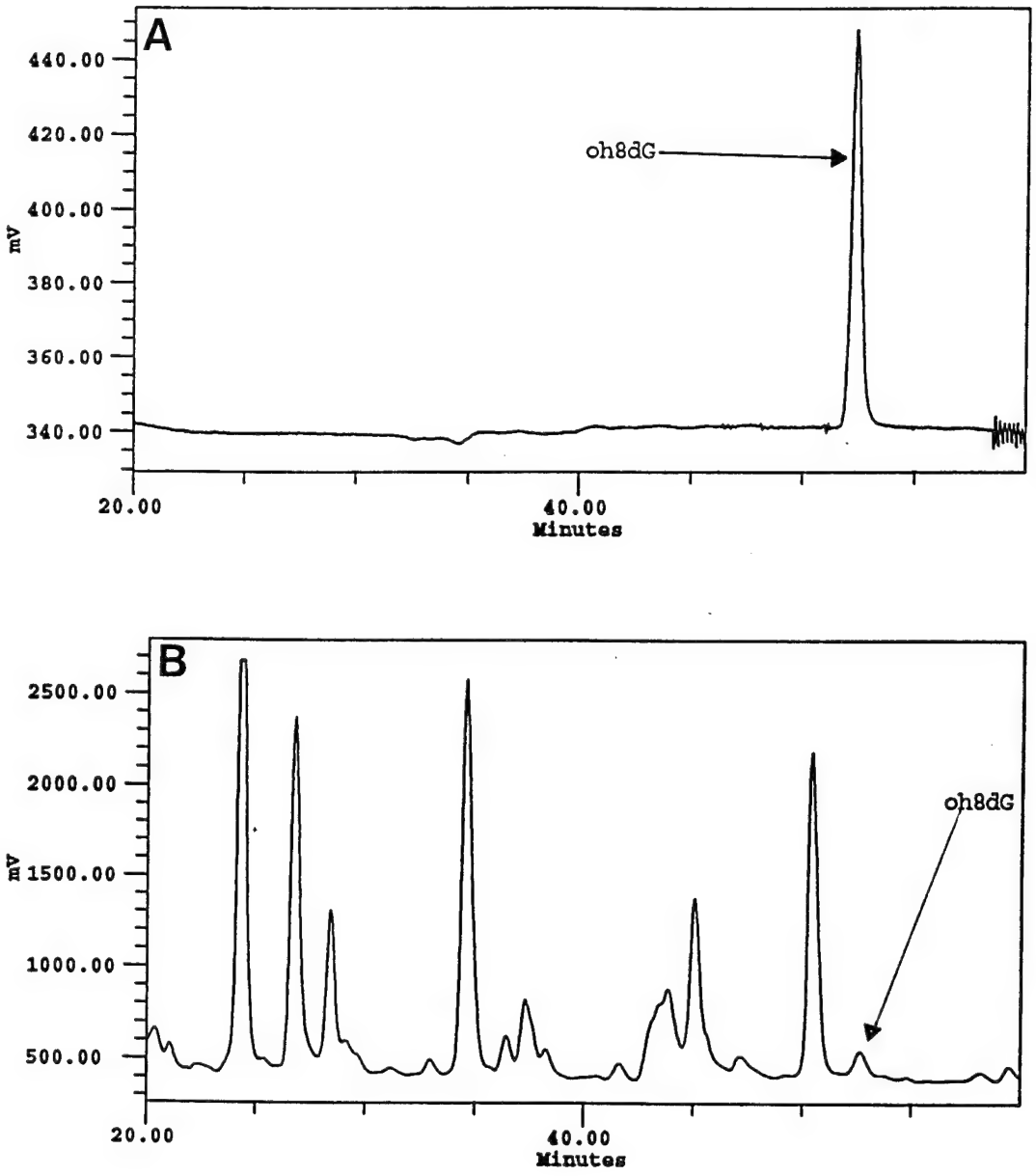


Figure 34A and 34B: A standard chromatograph for 8-hydroxy-2'-deoxyquanosine (oh8dG) standard is shown in panel A and a typical urine sample chromatograph in panel B. The EC detector was set at 0.6 mV. A two buffer gradient mobile phase was used as specified in the methods.

M. URINARY OH8DG ANALYSIS

Urinary oh8dG was analyzed by a modified method of Ames (1991). For urinary analysis, add 60 µl of urine to 540 µl of deionized water and vortex gently. Remove 100 µl of diluted urine for creatinine analysis. Creatinine was measure using a creatinine detection kit (SIGMA procedure No. 555) based on reaction of creatinine with acidic picrate. A COBAS MiraS spectrophotometer calibrated with standard creatinine solutions (SIGMA 925-3 and 925-15) was used to measure difference in color intensity at 500 nm before and after acidification of creatinine-picrate complex. The diluted urine was centrifuged at 12,000 x g for 10 minutes and analyzed by an HPLC-EC detector within 12 hrs. The HPLC system consisted of a Waters 600E controller and pump with a Waters 700 Satellite WISP autoinjector with series of 3 Waters Nova-Pak C18, 4 µ, 8 x 100 mm in a Radial-Pak cartridge with a Nova-Pak C18 Guard-Pak Insert eluted with a gradient consisting of two solvents: Solvent A (980 ml 25 mM potassium phosphate monobasic (pH=6.7), 14 ml acetonitrile and 6 ml methanol) and Solvent B(600 ml 25 mM potassium phosphate monobasic (pH=6.7), 280 ml acetonitrile and 120 ml methanol). The following gradient was used:

Time	Solvent A	Solvent B
0	100	0
30	94	6
45	94	6
60	60	40
70	60	40
75	100	0

Run time per sample was 90 minutes. A CC-5/LC-4C Amperometer Detector from BAS System (West Lafayette, IN) was set at 0.1 μA range, +600 mV potential, 0.1 filter for oh8dG analysis and a Waters 484 Tunable Absorbance Detector at 260 nm for dGuo analysis. The peak eluted at 52 minutes. Results were expressed as fmoles oh8dG per mg creatinine.

N. DNA S-PHASE SYNTHESIS

Pump and Tissue Preparation

One week prior to sacrifice, osmotic mini-pumps (Alza Company, Palo Alto, CA) containing ^{3}H thymidine (65-85 Ci/mmole; 0.5 mCi delivered per hour) were subcutaneously implanted into the five mice or five rats being sampled from each dose group for that time (Eldrige et al., 1990). At sampling, the livers were separated into lobes and each lobe cut into 2-4 mm

thick longitudinal strips, which were fixed in 10% neutral buffered formalin (100 ml 37-40% formaldehyde, 900 ml dH2O, 4 g sodium phosphate monobasic, and 6.5 g sodium phosphate dibasic) for 48 hours and processed for histologic analysis.

Tissue Processing

Tissue processing was accomplished using a Fisher Scientific LX-120 tissue processor. Tissue was washed processed sequentially for:

15 minutes in fresh formalin buffer

60 minutes in 70% ethanol

60 minutes in 80% ethanol

90 minutes in 95% ethanol

90 minutes in 95% ethanol (beibrich)

60 minutes in 100% ethanol (3 times)

180 minutes in Hemo D (2 times)

105 minutes in paraffin (2 times)

After processing, tissue was embedded on a Jung Histo Embeddor, sectioned to 5 microns using a Reichert Jung 2030 biocut and mounted on Fisherbrand Superfrost/Plus Microscopeslides.

Autordiography and DNA Synthesis Quantification

Slides were dipped in NTB2 photographic emulsion (Eastman Kodak, Rochester, NY), air dried, and stored at -20°C. After 12 weeks, slides were processed in Kodak D-19 developer and fixer. The autoradiograms were then stained with hematoxylin and eosin and a glass coverslip was mounted on top of the tissue with Permount. Slides were examined for replicative DNA synthesis according to Eldrige et al., (1990). A section of duodenum from the respective animal was included in each block to ensure administration of tritiated thymidine to each animal had occurred. Cells undergoing DNA synthesis were quantitated in all treatment groups. DNA synthesis was evaluated in total liver. One hundred fields (a minimum of 1000 hepatocytes) were evaluated for DNA synthesis. The labeling index was determined by dividing the total number of labeled cells (labeled nuclei) by the total number of hepatocytes counted x 100.

O. STATISTICAL ANALYSIS

The mean ± standard deviation was determined for each treatment group. Significant differences between control and experimental groups were detected by analysis of variance followed by Dunnett's t-test or Fisher's Least Significant Difference. (Gad and Weil, 1986).

IV. EXPERIMENTAL DESIGN AND RESULTS

A. Aim 1: Determine if differences exist among rodent species in the antioxidant/oxidant balance.

The basal levels of oxidative stress and antioxidants in mice and rats were measured to ascertain if a relationship exist between the rate of spontaneous hepatic tumor formation and the basal oxidative stress of these rodents. Using five animals per group, the C3H, B6C3F1, and C57Bl mice and the F344 rat were examined for basal levels of SOD, CAT, GSH-Px, GST, Vit E, Vit C, GSH and UA. Also, basal levels of oxidative stress as measured by oh8dG were recorded.

Hepatic enzymatic antioxidant levels are shown in Table 7. The F344 rat liver showed significantly lower GSH-Px and SOD activity than the mouse strains. No significant differences were observed among mice strains for these two enzymes. The level of CAT activity in the rat was intermediate to the mouse strains being significantly lower than the C3H mouse but significantly higher than the B6C3F1 and C57Bl mouse. Lastly, GST levels in the rat were less than half those seen in mice. Non-enzymatic antioxidant levels in the rodent liver are showed in Table 7. Basal levels of hepatic Vit C and Vit E were found to be significantly higher in F344 rats than in mouse strains. Both Vit C and E were three fold higher in the rat. In contrast, the

F344 rat's GSH level was one third that of the mice. No significant differences were observed in hepatic UA levels. Table 7 shows basal hepatic oh8dG levels for mice and rats. The rat had significantly lower levels of hepatic oh8dG than the C3H mouse; the C57Bl mouse also had significantly lower hepatic oh8dG than the C3H mouse.

Units of Enzyme Activity

GST: amount of enzyme that catalyzed formation of 1 μmole of S-2,4-dinitrophenyl-glutathione per minute at 30°C using 1 mM of GSH

SOD: amount of enzyme which inhibited auto oxidation of pyrogallol by 50%

CAT: k/0.00693 where k =[log{(absorbance of standard-absorbance of blank)/(absorbance of standard-absorbance of sample)}]*(2.3/time)

GSH-Px: μmoles NADPH oxidized per minute

Assay values were based on liver samples from five animals per group. Statistical analysis was based on an ANOVA of $P<0.05$ followed by the student's "t" test.

[a] significantly decreased ($p < 0.05$) compared to B6C3F1 or C57Bl/6 mice

[b] significantly decreased ($p < 0.05$) compared to B6C3F1, C57Bl/6, or C3H/He mice

[c] significantly increased ($p < 0.05$) compared to B6C3F1 or C57Bl/6 mice

[d] significantly decreased ($p < 0.05$) from C3H/He mice

[e] significantly increased ($p < 0.05$) compared to B6C3F1, C57Bl/6, or C3H/He mice

Table 7: Comparison of Hepatic Enzymatic and Non-enzymatic Antioxidant Activity in Mouse and Rat with Hepatic oh8dG Concentrations

Enzyme	C3H/He	B6C3F1	C57Bl/6	F344
SOD	17.9 ± 4.4	16.7 ± 1.6	16.4 ± 0.4	12.8 ± 1.5 [b]
GSH-Px	60.3 ± 0.8	57.9 ± 1.1	58.6 ± 9.9	41.7 ± 5.3 [b]
GST	0.35 ± 0.01	0.30 ± 0.02	0.41 ± 0.03	0.15 ± 0.01 [b]
CAT	652 ± 16 [c]	467 ± 16	475 ± 11	580 ± 56 [c,d]
GSH	6.53 ± 1.90	5.04 ± 0.87	6.18 ± 0.57	2.27 ± 0.63 [b]
Vit C	0.83 ± 0.09	0.84 ± 0.05	0.75 ± 0.20	2.34 ± 0.34 [e]
Vit E	1.54 ± 0.15	1.63 ± 0.30	1.32 ± 0.63	4.73 ± 1.16 [e]
UA	4.39 ± 0.54	5.61 ± 0.51	3.46 ± 1.00	4.88 ± 0.67
oh8dG (oh8dG/dGuo x 10^{-5})	7.86 ± 2.35	5.16 ± 1.31	3.24 ± 1.59 [d]	2.04 ± 0.67 [d]

B. Aim 2: Determine if dieldrin perturbs the hepatic antioxidant/oxidant balance in rodent hepatocytes.

Part 1: Does dieldrin produce ROS in rodent microsomes?

Part 2: Does dieldrin produce ROS in rodent primary hepatocyte cell cultures?

Part 3: Does dieldrin produce oxidative stress in rodent hepatocyte primary cell cultures?

Microsome characterization

Liver microsomes from both normal rodents and rodents given 10 mg dieldrin/kg diet for 14 days were analyzed for cyt P-450 activity. The level of P-450 activity increased in both mouse and rat microsomes after dieldrin treatment (Table 8). However, only a single isoenzyme showed a marked increase in rats; whereas, all three increased in mice. Three ether's of resorufin were used to characterize the activity of the microsomes: benzyl ether resorufin for cyt P-450 2B, methyl ether resorufin for cyt P-450 1A and ethyl ether resorufin for cyt P-450 1A1. An increase in resorufin produced by metabolism of the ethers by cytochrome P-450 was used to characterize the activity of the microsomes. Cyt P-450 1A1 activity increased 17 fold in mouse microsomes after 14 days on dieldrin diet but only 10 fold in the rat. However, while the mouse showed an 18 fold increase in Cyt P-450 2B and a 6 fold increase in Cyt P-450 1A, rat microsomes only showed a modest

increase of 1.5 to 2 fold over control. The control values in this table were comparable to those seen in the literature for the B6C3F1 mouse and the F344 rat (Lubet, et al. 1990).

Microsome ROS Detection

In order to determine if the microsomes were fully active during the course of the experiment, a time course was run with normal mouse microsomes using a dose of 25 μM dieldrin (Table 9). A steady increase in the 2,3-DHBA and 2,5-DHBA was observed ($r^2 = 0.962$; $P < 0.05$). The microsomes were fully active in this time period and showed no signs of inactivation. All other experiments were run at 30 minutes for consistency.

Mouse and rat microsomes were treated with dieldrin (0.1, 1, 5, 10, 25, and 50 μM) for 30 minutes. The aromatic hydroxylation products formed by ROS reaction with salicylate were then analyzed by HPLC (Floyd, et al. 1984; Grootveld and Halliwell, 1986). Microsomes from both untreated and dieldrin pretreated mice showed significant elevation of free radical production when dosed with dieldrin as indicated by an increase in 2,3-DHBA (Table 10). Dieldrin caused a 2 fold increase in 2,3-DHBA production in normal mouse microsomes and a three fold increase in microsomes from dieldrin pretreated mice. This increase reached a maximum at 5 μM dieldrin in normal mouse microsomes whereas the 2,3-DHBA adduct formed with

induced mouse microsomes did not plateau until a dose of 25 μM dieldrin was used. In contrast, normal and induced rat microsomes showed no significant increase in 2,3-DHBA formation. Dieldrin alone did not cause formation of 2,3-DHBA in reaction mixtures when microsomes were not present, indicating that dieldrin does not spontaneously produce radical species. Likewise, when using heat treated microsomes no free radicals were detected (data not shown). Surprisingly, the 2,5-DHBA adduct of salicylic acid significantly increased in both mouse and rat microsomes from both normal and dieldrin pretreated rodents (Table 11).

Hepatocyte ROS Detection

Initially cytotoxicity, determined by LDH release was performed as a range finding experiment to determine dosing for hepatocyte assays (Table 12). Doses of dieldrin greater than 50 μM could not be used due to precipitation of the dieldrin in the media. A 24 hour LDH assay showed no major differences between mouse and rat hepatocytes. Both species showed significant increases in LDH release above control at 50 μM dieldrin. The mouse also showed a significant increase in LDH at 25uM. Hepatocytes isolated from mice and rats, which had been given 10 mg/kg dietary dieldrin for 14 days, showed no significant increase above their respective controls.

Next, salicylate loading experiments were conducted to determine the best dose of salicylate to use and the length of time to pre-incubate

hepatocytes. Table 13 shows that 10 mM salicylic acid provided the higher level of incorporation in the hepatocytes as compared to 1 mM salicylic acid. By 60 minutes, the loading of 10 mM salicylic acid into hepatocytes had reached a plateau. These doses of salicylate were not cytotoxic to the hepatocytes. All other experiments were conducted using a 60 minute pre-incubation of 10 mM salicylic acid.

Hepatocytes were incubated with salicylic acid and dosed with dieldrin (0.1, 1, 5, 10, 25, and 50 μM) for 60 minutes. Freshly isolated hepatocytes were utilized to insure an active xenobiotic metabolism system (Skett, 1993). Mice and rats were placed on either NIH-07 diet or NIH-07 diet with 10 mg dieldrin/kg diet. After 14 days, hepatocytes were isolated from these rodents and dosed in vitro with dieldrin. Normal mouse hepatocytes showed a steady increase in free radical generation with dieldrin starting at 0.1 μM (Table 14). Mouse hepatocytes dosed with 25 μM and 50 μM dieldrin resulted in a two fold increase in 2,3-DHBA formation. Hepatocytes isolated from mice pretreated for 14 days on dieldrin diet showed a similar increase in 2,3-DHBA production (2.5 times control hepatocytes). Rat hepatocytes, normal or dieldrin-induced, showed no increase in 2,3-DHBA. Again, the 2,5-DHBA adduct of salicylic acid significantly increased in both mouse and rat hepatocytes from both normal and dieldrin pretreated rodents (Table 15).

Antioxidant/pro-oxidant balance in isolated hepatocytes

A 24, 48 and 72 hour LDH assay was run on mouse hepatocytes to determine the cytotoxicity of dieldrin (Table 16). The 25 μM and 50 μM dose groups were significantly above control at all time points with the 25 μM groups being 2 fold higher and the 50 μM groups being 3 fold higher (cumulative control = 10.3 %). A 24, 48 and 72 hour LDH assay was run on rat hepatocytes to determine the cytotoxicity of dieldrin (Table 17). The rat showed its largest increase in LDH release at 50 μM with a 3 fold increase over control (cumulative control = 9.04%). While several other dose groups were significant from control, the LDH values were still well below the 50% mark (LDH-50).

Mouse and rat primary culture hepatocytes were treated for 24 hours with 0.1, 1, 5, 10, 25 and 50 μM Dieldrin to measure MDA levels. The results are shown in table 18. The thiobarbituate assay showed an increase of MDA in mouse but not rat hepatocytes. A significant increase above control was observed in mouse hepatocytes at a dose of 25 and 50 μM dieldrin. No change was observed in rat hepatocytes. Mouse and rat primary culture hepatocytes were treated for 48 hours with 0.1, 1, 5, 10, 25 and 50 μM Dieldrin. oh8dG levels are shown in table 19 for mouse and rat hepatocytes treated 48 hours with dieldrin. An increase in oh8dG levels were observed in mouse but not rat hepatocytes. A significant increase above control was observed in mouse hepatocytes at a dose of 10 and 25 μM dieldrin.

It was desired to measure the antioxidant levels in cultured hepatocytes. The values for Vit E, Vit C and GSH are shown in table 20 after a 24 hour dosing with dieldrin. Vit E levels decreased in a dose responsive manner in both mouse and rat hepatocytes while ascorbic acid increased. The decrease in Vit E was significantly below control at doses of 1 μM dieldrin and above for both mouse and rat hepatocytes. While both groups decreased to about 20% of their original values, the rat consistently had about twice the amount of Vit E as the mouse. Vit C levels in mouse and rat hepatocytes showed an opposite trend to Vit E. Vit C levels in both mouse and rat hepatocytes steadily increased. In mice, 0.1 μM dieldrin and above were significantly increased over control. In the rat, 5 μM and above were significantly increased over control. Vit E and Vit C were both higher in rat controls than in mouse control hepatocytes. Surprisingly, GSH only increased in mouse hepatocytes, being significantly above control levels at a dose of 25 and 50 μM dieldrin. The rat showed no such increase. In contrast to Vit E and Vit C, the mouse had higher levels of GSH than did the rat.

In summary, aim two showed the mouse, but not the rat, to produce free radicals in both microsomal preparations and whole cells. This production of free radicals resulted in oxidative damage to mouse hepatocytes as indicated by increased MDA and oh8dG concentrations.

Table 8: Cytochrome P-450 Activity of Microsomes for B6C3F1 Mouse and F344 Rat

	B6C3F1 Mouse		F344 Rat	
	control	dieldrin	control	dieldrin
Resorufin Benzyl Ether	5.69	103.20	18.11	38.23
Resorufin Ethyl Ether	22.77	401.92	30.78	309.51
Resorufin Methyl Ether	44.12	249.84	23.54	36.41
mg microsome protein per gram liver	48.6	51.6	56.9	55.2

Values reflect pooled samples of microsomes from 3 mice or 2 rats. Mice and rats were 6-8 weeks of age. Animals were placed on diets of either NIH-07 or NIH-07 supplemented with 10 mg/kg dieldrin prior to isolation of microsomes. Value for control rodents were consistent with those reported in the literature (Lubet, et al. 1990). Value for microsome activity are expressed as nM/mim/mg protein.

Table 9: Time Course of Dieldrin ROS Production in B6C3F1 Mouse Microsomes

Time (minutes)	2,3-DHBA	2,5-DHBA
0	0 0	0 0
5	0.032 ±0.004	0.491 ±0.256
10	0.067 ±0.008	1.399 ±0.188
20	0.093 ±0.004	1.993 ±0.158
30	0.124 ±0.002	2.700 ±0.181
45	0.149 ±0.019	3.505 ±0.234
60	0.180 ±0.022	4.221 ±0.145
r^2	0.962	0.968

Values are expressed as mean ± standard deviation based on three samples per group. Mice and rats were 6-8 weeks of age. Animals were placed on a diet of NIH-07 for 14 days prior to microsome isolation. Statistical analysis was accomplished by using linear regression analysis with $P<0.05$. Values are expressed as either (2,5-DHBA/SA) x 100 or (2,5-DHBA/SA) x 100.

Table 10: 2,3-dihydroxybenzoic Acid Production in B6C3F1 Mouse and F344 Rat Microsomes

Dieldrin Dose (μM)	normal mouse	normal rat	induced mouse	induced rat
0	0.029 ±0.001	0.0047 ±0.0005	0.028 ±0.002	0.016 ±0.001
0.1	0.032 ±0.002	0.0040 ±0.0005	0.030 ±0.003	0.014 ±0.003
1.0	0.047 ±0.002	0.0047 ±0.0004	0.054 ±0.007*	0.021 ±0.002
5.0	0.057 ±0.005*	0.0051 ±0.0012	0.072 ±0.004*	0.022 ±0.001
10.0	0.058 ±0.001*	0.0060 ±0.0018	0.079 ±0.005*	0.021 ±0.003
25.0	0.060 ±0.004*	0.0057 ±0.0007	0.086 ±0.004*	0.019 ±0.001
50.0	0.059 ±0.004*	0.0037 ±0.0002	0.087 ±0.005*	0.022 ±0.001

Values are expressed as mean ± standard deviation based on three samples per group. Mice and rats were 6-8 weeks of age. Animals were placed on diets of either NIH-07 or NIH-07 supplemented with 10 mg/kg dieldrin for 14 days prior to microsome isolation. A 30 minute microsomal reaction period was used for assay. Statistical analysis was accomplished by first using ANOVA with $P<0.05$ followed by the post hoc "t" test. * indicates significance $P<0.05$ when compared to control. Values are expressed as (2,3-DHBA/SA) x 100.

Table 11: 2,5-Dihydroxybenzoic Acid Production in B6C3F1 Mouse and F344 Rat Microsomes

Dieldrin Dose (μM)	normal mouse	normal rat	induced mouse	induced rat
0	0.020 ±0.006	0.057 ±0.012	0.419 ±0.046	0.053 ±0.020
0.1	0.041 ±0.007*	0.052 ±0.011	0.454 ±0.030	0.121 ±0.038
1.0	0.220 ±0.016*	0.384 ±0.069*	1.101 ±0.110*	0.372 ±0.220
5.0	0.406 ±0.007*	0.601 ±0.056*	1.705 ±0.100*	0.570 ±0.115*
10.0	0.572 ±0.035*	0.603 ±0.016*	1.945 ±0.151*	0.587 ±0.165*
25.0	0.472 ±0.017*	0.599 ±0.044*	1.982 ±0.142*	0.547 ±0.077*
50.0	0.492 ±0.011*	0.577 ±0.049*	1.867 ±0.200*	0.526 ±0.124*

Values are expressed as mean ± standard deviation based on three samples per group. Mice and rats were 6-8 weeks of age. Animals were placed on diets of either NIH-07 or NIH-07 supplemented with 10 mg/kg dieldrin for 14 days prior to microsome isolation. A 30 minute microsomal reaction period was used for assay. Statistical analysis was accomplished by first using ANOVA with $P<0.05$ followed by the post hoc "t" test. * indicates significance $P<0.05$ when compared to control. Values are expressed as (2,5-DHBA/SA) x 100.

Table 12: Cytotoxicity for Mouse and Rat Hepatocytes after a 24 hour Dieldrin Treatment

Dieldrin Dose (μM)	normal mouse	normal rat	induced mouse	induced rat
0	3.89 ±0.85	3.37 ±0.51	6.19 ±0.69	10.66 ±0.75
0.1	4.12 ±0.44	2.70 ±0.21	6.35 ±0.57	11.94 ±0.27
1.0	4.08 ±0.56	2.90 ±0.64	5.99 ±0.79	10.97 ±0.51
5.0	4.47 ±0.54	3.63 ±0.77	5.39 ±1.00	10.92 ±0.85
10.0	4.86 ±0.22	3.97 ±0.47	6.24 ±1.52	9.33 ±1.03
25.0	6.50 ±0.70*	4.11 ±0.18	8.26 ±1.15	9.97 ±0.84
50.0	10.18 ±1.22*	10.26 ±0.69*	7.32 ±0.82	12.25 ±1.40

Values are expressed as mean ± standard deviation based on three samples per group. Mice and rats were 6-8 weeks of age. Animals were placed on diets of either NIH-07 or NIH-07 supplemented with 10 mg/kg dieldrin for two weeks prior to hepatocyte isolation. Statistical analysis was accomplished by first using ANOVA with $P<0.05$ followed by the post hoc "t" test. * indicates significance $P<0.05$ when compared to control.

Table 13: Salicylate Loading of Mouse Hepatocytes

Time (Hours)	1 mM Salicylic Acid (ug/million cells)	10 mM Salicylic Acid (ug/million cells)
Loading		
0.25	5.20 ±0.88	16.51 ±1.55
0.50	8.65 ±0.42	22.12 ±2.06
1.00	9.82 ±0.51	28.74 ±1.64
2.00	15.71 ±1.39	30.52 ±0.99

In the loading experiments of salicylic acid into mouse hepatocytes, hepatocytes were treated with either 1 or 10 mM salicylic acid in DMEM/F-12 media. After the stated time period, hepatocytes were quickly washed two times with 1 ml of PBS/G. 1 ml of 5% trichloroacetic acid in PBS/G was then added to hepatocytes. Cells were scraped off of culture dish, centrifuged and supernatant assayed for salicylate content. Values are expressed as mean ± standard deviation based on three samples per group.

Table 14: 2,3-dihydroxybenzoic Acid Production in B6C3F1 Mouse and F344 Rat Hepatocytes

Dieldrin Dose (μM)	normal mouse	normal rat	induced mouse	induced rat
0	0.065 ±0.012	0.133 ±0.008	0.047 ±0.009	0.040 ±0.003
0.1	0.068 ±0.010	0.158 ±0.026	0.046 ±0.005	0.061 ±0.023
1.0	0.096 ±0.005*	0.138 ±0.068	0.049 ±0.021	0.071 ±0.020
5.0	0.080 ±0.036	0.093 ±0.022	0.061 ±0.016	0.027 ±0.004
10.0	0.095 ±0.019*	0.085 ±0.034	0.082 ±0.016	0.047 ±0.030
25.0	0.140 ±0.024*	0.082 ±0.020	0.118 ±0.009*	0.037 ±0.013
50.0	0.136 ±0.021*	0.093 ±0.003	0.113 ±0.008*	0.040 ±0.012

Values are expressed as mean ± standard deviation based on three samples per group. Mice and rats were 6-8 weeks of age. Animals were placed on diets of either NIH-07 or NIH-07 supplemented with 10 mg/kg dieldrin for two weeks prior to hepatocyte isolation. Statistical analysis was accomplished by first using ANOVA with $P<0.05$ followed by the post hoc "t" test. * indicates significance $P<0.05$ when compared to control. Values are expressed as (2,3-DHBA/SA) x 100.

Table 15: 2,5-dihydroxybenzoic Acid Production in B6C3F1 Mouse and F344 Rat Hepatocytes

Dieldrin Dose (μM)	normal mouse	normal rat	induced mouse	induced rat
0	0.055 ±0.006	0.396 ±0.324	0.244 ±0.029	0.305 ±0.025
0.1	0.101 ±0.011*	0.406 ±0.022	0.239 ±0.032	0.408 ±0.065
1.0	0.089 ±0.014*	1.430 ±0.163*	0.348 ±0.049*	0.563 ±0.036*
5.0	0.199 ±0.020*	1.461 ±0.308*	0.603 ±0.169*	0.840 ±0.133*
10.0	0.469 ±0.210*	1.558 ±0.291*	0.693 ±0.117*	0.892 ±0.069*
25.0	0.520 ±0.063*	1.346 ±0.128*	1.064 ±0.122*	0.899 ±0.190*
50.0	0.590 ±0.105*	1.358 ±0.230*	1.107 ±0.144*	1.088 ±0.202*

Values are expressed as mean ± standard deviation based on three samples per group. Mice and rats were 6-8 weeks of age. Animals were placed on diets of either NIH-07 or NIH-07 supplemented with 10 mg/kg dieldrin for two weeks prior to hepatocyte isolation. Statistical analysis was accomplished by first using ANOVA with $P<0.05$ followed by the post hoc "t" test. * indicates significance $P<0.05$ when compared to control. Values are expressed as (2,5-DHBA/SA) x 100.

Table 16: Cytotoxicity Assay for B6C3F1 Mouse Hepatocytes Following 24, 48 and 72 Hours of Dieldrin Treatment

Dieldrin Dose (μM)	24 Hour	48 Hour	72 Hour	Total
0	3.89 ±0.85	3.05 ±1.15	3.36 ±1.25	10.30 ±3.21
0.1	4.12 ±0.44	3.31 ±0.63	2.88 ±0.65	10.31 ±1.33
1.0	4.08 ±0.56	3.59 ±0.99	3.10 ±1.15	10.77 ±2.21
5.0	4.47 ±0.54	3.03 ±0.53	3.10 ±0.59	10.59 ±1.24
10.0	4.86 ±0.22	3.86 ±0.40	3.91 ±0.31	12.63 ±0.33
25.0	6.50 ±0.70*	7.15 ±0.75*	6.49 ±0.68*	20.13 ±2.12*
50.0	10.18 ±1.22*	12.24 ±0.69*	14.18 ±1.14*	36.60 ±2.65*

Values of LDH release are expressed as mean ± standard deviation based on three samples per dose group. Mice and rats were 6-8 weeks of age. Animals were placed on diets of NIH-07 for one week prior to hepatocyte isolation. Statistical analysis was accomplished by first using ANOVA with $P<0.05$ followed by the post hoc "t" test. * indicates significance $P<0.05$ when compared to control.

Table 17: Cytotoxicity Assay for F344 Rat Hepatocytes Following 24, 48 and 72 Hours of Dieldrin Treatment

Dieldrin Dose (μM)	24 Hour	48 hour	72 hour	Total
0	3.37 ±0.51	3.14 ±0.47	2.53 ±0.55	9.04 ±0.58
0.1	2.70 ±0.21	3.19 ±0.46	3.05 ±0.54	8.94 ±1.20
1.0	2.90 ±0.64	3.14 ±0.75	2.84 ±0.36	8.88 ±1.73
5.0	3.63 ±0.77	4.35 ±0.47*	3.20 ±0.63	11.18 ±0.93*
10.0	3.97 ±0.47	4.30 ±0.13*	4.34 ±0.55*	12.61 ±0.07*
25.0	4.11 ±0.18	4.39 ±0.42*	4.42 ±0.51*	12.92 ±0.48*
50.0	10.26 ±0.69*	10.67 ±1.15*	7.80 ±0.58*	28.73 ±1.36*

Values of LDH release are expressed as mean ± standard deviation based on three samples per dose group. Mice and rats were 6-8 weeks of age. Animals were placed on diets of NIH-07 for one week prior to hepatocyte isolation. Statistical analysis was accomplished by first using ANOVA with $P<0.05$ followed by the post hoc "t" test. * indicates significance $P<0.05$ when compared to control.

Table 18: Lipid Peroxidation Assay for B6C3F1 Mouse and F344 Rat Hepatocytes with Dieldrin Dosing

Dieldrin Dose (μM)	Mouse MDA (nmole/mg protein)	Rat MDA (nmole/mg protein)
0	0.54 ±0.022	0.49 ±0.06
0.1	0.54 ±0.042	0.58 ±0.06
1.0	0.55 ±0.037	0.61 ±0.08
5.0	0.53 ±0.084	0.67 ±0.08
10.0	0.62 ±0.075	0.60 ±0.11
25.0	0.76 ±0.154*	0.48 ±0.09
50.0	0.96 ±0.105*	0.55 ±0.09

MDA values are expressed as mean ± standard deviation based on three samples per group using the thiobarbituric acid assay. Mice and rats were 6-8 weeks of age. Animals were placed on diets of NIH-07 for one week prior to hepatocyte isolation. Hepatocytes were treated for 24 hours before analysis. Statistical analysis was accomplished by first using ANOVA with $P<0.05$ followed by the post hoc "t" test. * indicates significance $P<0.05$ when compared to control.

Table 19: 8-hydroxy-2'-deoxyguanosine for B6C3F1 Mouse and F344 Rat Hepatocytes with Dieldrin Dosing

Dieldrin Dose	Mouse (oh8dG/dGuo x 10^{-5})	Rat (oh8dG/dGuo x 10^{-5})
0	6.96 ±0.37	2.75 ±1.24
0.1	7.05 ±1.50	3.53 ±1.61
1.0	8.94 ±2.05	4.08 ±2.33
5.0	8.92 ±1.80	3.26 ±1.31
10.0	12.93 ±1.54*	3.69 ±1.32
25.0	26.44 ±9.99*	4.72 ±1.57
50.0	9.8 ±2.96	3.73 ±0.63

Values are expressed as mean ± standard deviation based on three samples per group. Mice and rats were 6-8 weeks of age. Animals were placed on diets of NIH-07 for one week prior to hepatocyte isolation. Hepatocytes were treated for 48 hours before analysis. Statistical analysis was accomplished by first using ANOVA with $P<0.05$ followed by the post hoc "t" test. * indicates significance $P<0.05$ when compared to control.

Table 20: Vit E, Vit C and GSH Levels for B6C3F1 Mouse and F344 Rat Hepatocytes with Dieldrin Dosing

Dieldrin Dose (μM)	Vit E (pmoles/mg protein)		Vit C (nmoles/mg protein)		GSH (nmoles/mg protein)	
	Mouse	Rat	Mouse	Rat	Mouse	Rat
0	11.10 ±0.81	21.54 ±1.83	0.220 ±0.019	0.25 ±0.01	4.20 ±0.34	2.85 ±0.37
0.1	10.60 ±1.99	20.25 ±0.72	0.422 ±0.012*	0.34 ±0.02	5.12 ±1.75	3.17 ±0.27
1.0	9.31 ±2.50*	11.85 ±0.89*	0.591 ±0.052*	1.28 ±0.59	6.24 ±2.60	2.85 ±0.39
5.0	7.81 ±1.89*	9.23 ±0.34*	0.644 ±0.054*	2.15 ±0.19*	5.96 ±2.04	2.98 ±0.06
10.0	4.45 ±1.10*	6.88 ±0.48*	0.845 ±0.130*	3.34 ±0.26*	7.48 ±3.52	2.20 ±0.10
25.0	3.31 ±0.84*	4.88 ±0.34*	1.115 ±0.139*	5.22 ±0.97*	11.52 ±2.71*	1.87 ±0.08
50.0	2.40 ±1.33*	6.00 ±0.73*	1.348 ±0.220*	5.26 ±0.98*	17.32 ±1.67*	1.98 ±0.16

Values are expressed as mean ± standard deviation based on three samples per group. Mice and rats were 6-8 weeks of age. Animals were placed on diets of NIH-07 for one week prior to hepatocyte isolation. Hepatocytes were treated for 24 hours before analysis. Statistical analysis was accomplished by first using ANOVA with $P<0.05$ followed by the post hoc "t" test. * indicates significance $P<0.05$ when compared to control.

Aim 3: Determine if dieldrin produces hepatic oxidative stress in subchronically treated rats and mice.

Dieldrin was mixed with NIH-07 diet and formulated into pellets by a commercial supplier (Dyets, Inc. Bethlehem, PA). There were four dose groups, including control, consisting of 0, 0.1, 1 and 10 mg dieldrin/kg diet (based on Walker, et al. 1972). Five animals were used per dose group. Animals were placed on diet and were sacrificed on days 7, 14, 28, and 90 days based on *in vivo* DNA synthesis studies conducted by Stevenson, et al. (1995).

No significant change in body weight was seen in dieldrin treated mice or rats compared to controls (0.0 mg dieldrin/kg diet) in any dose group or time point examined(Tables 21). At the highest dose studied (10 mg dieldrin/kg diet) a significant increase in liver weight and the relative liver weight in mice was seen after 14, 28 and 90 days of treatment(Tables 22). No difference from control was seen in relative liver weights at any of the concentrations of dieldrin examined in the rat nor in the 1.0 and 0.1 mg dieldrin/kg diet treatment in the mice (Tables 23).

Hepatic DNA synthesis as measured by autoradiography showed a dieldrin dose related increase in mice (Table 24). No increase in DNA synthesis was seen from controls at any of the dieldrin concentrations examined in the rat. In contrast, mice showed an increase in DNA synthetic

labeling after 7 days to 4.4% at 10 mg/kg and 2.6% at 1.0 mg/kg. The labeling index appeared to plateau after 14 days of exposure (1 mg/kg = 2.35% and 10 mg/kg = 10.7%) and returned to near basal levels by day 90. At all time points the 10 mg/kg dieldrin diet produced a significant increase above controls in mice.

molecular antioxidant status

Both the mouse and the rat showed a significant decrease in Vit E levels with dieldrin (Table 25). Both species showed an initial ~50% decrease in hepatic Vit E levels with dieldrin but only the mouse showed a clean dose response on days 7 and 14 which correlated with dietary dieldrin ($r^2 = 0.529$, $P < 0.0003$ and $r^2 = 0.347$, $P < 0.0063$ respectively). Interestingly, the rat's basal hepatic Vit E was approximately three times that of the mouse. This may afford the rat better protection against ROS. Serum Vit E levels (Table 26), while slightly higher in the rat, were reduced in both species with dieldrin treatment. All dieldrin dose groups were significantly decreased below control values for both species.

Hepatic Vit C increased steadily in mice at 10 mg dieldrin/kg diet, increasing almost 4 fold over original values at day 7 (Table 27). Rats also showed a significant increase in Vit C at 10 mg dieldrin/kg diet (2 fold over control), but it was not as dramatic as in mice and did not continue to

increase with treatment. Similar to Vit E, the rat's control hepatic Vit C levels were about twice those of the mouse.

Hepatic GSH showed a significant increase above control for all dieldrin dose groups in mice at day 14 (Table 28). By day 28, only the 0.1 mg dieldrin/kg dose group was still elevated above control. The rat showed no clear overall pattern in hepatic GSH over dose or time. The mouse's control hepatic GSH was higher than those of the rat at all time points. UA also showed a significant increase above control in mice at day 14 for all dieldrin dose groups; the rat showed no such increase at any time point or dose (Table 29).

oxidative stress status

Hepatic MDA showed an initial increase in mice at days 7 and 14 which correlated to dietary dieldrin (r^2 =0.218, P <0.0381 and r^2= 405, P<0.0026 respectively) but no change was observed in the rat (Table 30). The decrease at later time points may have been a result of the increased hepatic Vit C and the burst of hepatic GSH at day 14. Rats showed no increase in hepatic MDA at any time or dose points. Urinary MDA, while not organ specific, is useful to non evasively monitor the status of the rodents during the course of the study. Mouse urinary MDA was higher than control at all time points and doses (Table 31). Rat urinary MDA showed no change until day 90 when it increased noticeably. This did not correlate with any

hepatic change in the rat and is remarked on now as a matter of record until further analysis is possible.

oh8dG is a useful marker for oxidative damage to DNA. Surprisingly, no change was observed in hepatic oh8dG in mice or rats (Table 32). However, a clear increase was seen in mouse urine (Table 33) which was parallel to the observed increase in DNA synthesis, suggesting that while oxidative damage did occur it was being repaired during the proliferative cycle.

In summary, aim three showed the mouse, but not the rat, to induce DNA S-phase synthesis. This induction of DNA S-phase synthesis coincided with a decrease in hepatic Vit E. It also coincided with an increase in hepatic MDA, urinary MDA and urinary oh8dG.

Table 21: 7, 14, 28 and 90 Day Body Weights for B6C3F1 Mice and F344 Rats in Subchronic Dieldrin Study

Dieldrin (mg/kg diet)	Sampling Time (days)	Body Weight (grams)	
		Mouse	Rat
0.0	7	24.04 ±2.30	203.68 ±7.96
0.1	7	25.53 ±1.25	204.35 ±6.98
1.0	7	25.12 ±0.88	200.75 ±4.18
10.0	7	25.13 ±1.47	206.90 ±3.73
0.0	14	23.88 ±1.64	229.15 ±11.58
0.1	14	25.01 ±1.01	225.59 ±11.08
1.0	14	25.35 ±0.91	233.16 ±12.23
10.0	14	27.16 ±0.71	239.56 ±4.97
0.0	28	26.60 ±2.18	259.90 ±17.18
0.1	28	25.75 ±1.55	269.54 ±9.98
1.0	28	27.24 ±1.81	284.41 ±22.34
10.0	28	26.45 ±1.34	274.52 ±11.77
0.0	90	30.20 ±2.37	348.89 ±8.60
0.1	90	29.70 ±1.75	382.49 ±17.30
1.0	90	30.60 ±2.21	379.35 ±12.44
10.0	90	32.23 ±1.66	367.78 ±14.01

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc "t" test. * indicates value significantly different from that of control (no dieldrin).

Table 22: 7, 14, 28 and 90 Day Liver Weights for B6C3F1 Mice and F344 Rats in Subchronic Dieldrin Study

Dieldrin (mg/kg diet)	Sampling Time (days)	Liver Weights (grams)	
		Mouse	Rat
0.0	7	1.70 ±0.21	9.67 ±0.22
0.1	7	1.63 ±0.08	9.08 ±0.39
1.0	7	1.68 ±0.08	9.80 ±0.43
10.0	7	1.81 ±0.14	10.78 ±0.37
0.0	14	1.43 ±0.12	11.37 ±0.69
0.1	14	1.50 ±0.07	11.17 ±0.73
1.0	14	1.48 ±0.14	11.61 ±0.90
10.0	14	2.00 ±0.24*	12.62 ±0.67
0.0	28	1.56 ±0.15	11.70 ±1.17
0.1	28	1.52 ±0.17	11.17 ±0.75
1.0	28	1.60 ±0.14	12.85 ±1.03
10.0	28	1.94 ±0.08*	13.30 ±0.62
0.0	90	1.76 ±0.17	12.83 ±0.76
0.1	90	1.56 ±0.08	14.35 ±0.68
1.0	90	1.82 ±0.21	14.19 ±0.48
10.0	90	2.50 ±0.21*	14.21 ±1.03

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc "t" test. * indicates value significantly different from that of control (no dieldrin).

Table 23: 7, 14, 28 and 90 Day Percent Liver Weight/Body Weight for B6C3F1 Mice and F344 Rats in Subchronic Dieldrin Study

Dieldrin (mg/kg diet)	Sampling Time (days)	LW/BW X100	
		Mouse	Rat
0.0	7	7.05 ±0.52	4.75 ±0.14
0.1	7	6.40 ±0.35	4.44 ±0.15
1.0	7	6.70 ±0.23	4.88 ±0.17
10.0	7	7.20 ±0.45	5.21 ±0.25
0.0	14	6.01 ±0.23	4.97 ±0.31
0.1	14	6.01 ±0.17	4.95 ±0.22
1.0	14	5.85 ±0.35	4.98 ±0.28
10.0	14	7.35 ±0.75*	5.27 ±0.23
0.0	28	5.88 ±0.37	4.49 ±0.19
0.1	28	5.89 ±0.39	4.15 ±0.29
1.0	28	5.87 ±0.24	4.52 ±0.09
10.0	28	7.32 ±0.31*	4.84 ±0.12
0.0	90	5.83 ±0.25	3.68 ±0.21
0.1	90	5.25 ±0.25	3.75 ±0.14
1.0	90	5.95 ±0.40	3.74 ±0.20
10.0	90	7.76 ±0.37*	3.86 ±0.17

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc "t" test. * indicates value significantly different from that of control (no dieldrin).

Table 24: 7, 14, 28 and 90 Day Hepatic DNA S-Phase Synthesis for B6C3F1 Mice and F344 Rats in Subchronic Dieldrin Study

Dieldrin (mg/kg diet)	Sampling Time (days)	DNA S-Phase Synthesis % labeled nuclei	
		Mouse	Rat
0.0	7	1.54 ±0.50	1.84 ±0.77
0.1	7	1.42 ±0.52	1.28 ±0.32
1.0	7	2.57 ±0.84*	2.20 ±0.97
10.0	7	4.43 ±1.14*	1.17 ±1.21
0.0	14	1.17 ±0.34	1.06 ±0.35
0.1	14	1.66 ±0.62	1.26 ±0.51
1.0	14	2.35 ±0.42*	1.11 ±0.31
10.0	14	10.72 ±3.38*	1.06 ±0.24
0.0	28	2.55 ±0.52	1.89 ±0.49
0.1	28	2.83 ±0.88	2.66 ±0.47
1.0	28	4.44 ±1.25*	2.10 ±0.20
10.0	28	10.41 ±2.44*	4.65 ±1.15
0.0	90	1.89 ±0.49	1.31 ±0.43
0.1	90	2.66 ±0.47*	1.31 ±0.43
1.0	90	2.10 ±0.20	1.39 ±0.31
10.0	90	4.65 ±1.16*	1.61 ±10.38

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc "t" test. * indicates value significantly different from that of control (no dieldrin).

Table 25: 7, 14, 28 and 90 Day Hepatic Vit E Concentrations for B6C3F1 Mice and F344 Rats in Subchronic Dieldrin Study

Dieldrin (mg/kg diet)	Sampling Time (days)	Hepatic Vit E (μmoles/g tissue)	
		Mouse	Rat
0.0	7	0.99 ±0.04	3.63 ±0.52
0.1	7	0.76 ±0.12*	1.83 ±0.63*
1.0	7	0.67 ±0.04*	1.83 ±0.47*
10.0	7	0.49 ±0.15*	1.82 ±0.66*
0.0	14	1.08 ±0.06	3.62 ±0.52
0.1	14	0.76 ±0.09*	2.79 ±0.28*
1.0	14	0.71 ±0.07*	2.67 ±0.30*
10.0	14	0.60 ±0.12*	2.63 ±0.49*
0.0	28	1.16 ±0.14	3.44 ±0.51
0.1	28	0.85 ±0.08*	2.92 ±0.18*
1.0	28	0.77 ±0.05*	3.15 ±0.27
10.0	28	0.87 ±0.08*	2.55 ±0.41
0.0	90	1.04 ±0.09	3.19 ±0.15
0.1	90	0.65 ±0.05*	1.99 ±0.26*
1.0	90	0.53 ±0.05*	2.12 ±0.16*
10.0	90	0.47 ±0.04*	2.24 ±0.26*

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc "t" test. * indicates value significantly different from that of control (no dieldrin).

Table 26: 7, 14, 28 and 90 Day Serum Vit E Concentrations for B6C3F1 Mice and F344 Rats in Subchronic Dieldrin Study

Dieldrin (mg/kg diet)	Sampling Time (days)	Serum Vit E (μmoles/ml serum)	
		Mouse	Rat
0.0	7	0.72 ±0.02	1.30 ±0.15
0.1	7	0.51 ±0.06*	0.89 ±0.19*
1.0	7	0.45 ±0.07*	0.86 ±0.21*
10.0	7	0.40 ±0.07*	0.99 ±0.09*
0.0	14	0.72 ±0.02	1.19 ±0.08
0.1	14	0.51 ±0.06*	0.85 ±0.08*
1.0	14	0.45 ±0.07*	0.86 ±0.10*
10.0	14	0.40 ±0.07*	0.81 ±0.14*
0.0	28	0.62 ±0.05	1.19 ±0.10
0.1	28	0.32 ±0.02*	0.71 ±0.11*
1.0	28	0.33 ±0.03*	0.71 ±0.13*
10.0	28	0.35 ±0.04*	0.83 ±0.05*
0.0	90	0.69 ±0.03	1.17 ±0.29
0.1	90	0.32 ±0.04*	0.76 ±0.17*
1.0	90	0.33 ±0.04*	0.86 ±0.19
10.0	90	0.34 ±0.03*	0.85 ±0.09

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc "t" test. * indicates value significantly different from that of control (no dieldrin).

Table 27: 7, 14, 28 and 90 Day Hepatic Vit C Concentrations for B6C3F1 Mice and F344 Rats in Subchronic Dieldrin Study

Dieldrin (mg/kg diet)	Sampling Time (days)	Hepatic Vit C (μmoles/g tissue)	
		Mouse	Rat
0.0	7	1.41 ±0.36	2.07 ±0.98
0.1	7	1.29 ±0.21	2.42 ±0.32
1.0	7	1.44 ±0.26	2.42 ±0.76
10.0	7	2.43 ±0.37*	3.10 ±0.92
0.0	14	1.03 ±0.30	2.54 ±1.39
0.1	14	1.58 ±0.37*	2.85 ±0.43
1.0	14	1.8 ±0.46*	3.42 ±0.83
10.0	14	3.37 ±0.39*	5.98 ±0.63*
0.0	28	1.35 ±0.31	2.14 ±0.29
0.1	28	1.07 ±0.23	2.21 ±0.22
1.0	28	1.64 ±0.13	2.21 ±0.51
10.0	28	5.01 ±0.33*	3.69 ±0.34*
0.0	90	1.18 ±0.26	2.26 ±0.90
0.1	90	0.85 ±0.20	1.77 ±0.55
1.0	90	1.42 ±0.42	2.41 ±0.99
10.0	90	5.89 ±0.98*	3.92 ±0.77*

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc "t" test. * indicates value significantly different from that of control (no dieldrin).

Table 28: 7, 14, 28 and 90 Day Hepatic GSH Concentrations for B6C3F1 Mice and F344 Rats in Subchronic Dieldrin Study

Dieldrin (mg/kg diet)	Sampling Time (days)	Hepatic GSH (μmoles/g tissue)	
		Mouse	Rat
0.0	7	5.82 ±0.86	4.03 ±1.40
0.1	7	6.16 ±1.34	5.72 ±0.59
1.0	7	5.57 ±1.71	4.71 ±1.31
10.0	7	5.65 ±0.94	3.75 ±1.35
0.0	14	5.86 ±1.30	3.85 ±0.66
0.1	14	9.49 ±3.32*	4.04 ±0.35
1.0	14	9.08 ±1.78*	4.70 ±0.48*
10.0	14	8.90 ±0.22*	3.85 ±0.45
0.0	28	5.66 ±0.74	4.52 ±0.92
0.1	28	7.50 ±0.68*	5.86 ±0.60*
1.0	28	6.57 ±0.54	4.71 ±0.62
10.0	28	5.11 ±0.40	3.60 ±0.41
0.0	90	5.14 ±0.95	4.22 ±0.16
0.1	90	5.40 ±0.70	4.67 ±0.69
1.0	90	5.21 ±0.75	4.55 ±0.68
10.0	90	4.54 ±0.78	4.84 ±0.72

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc "t" test. * indicates value significantly different from that of control (no dieldrin).

Table 29: 7, 14, 28 and 90 Day Hepatic UA Concentrations for B6C3F1 Mice and F344 Rats in Subchronic Dieldrin Study

Dieldrin (mg/kg diet)	Sampling Time (days)	Hepatic UA (μmoles/g tissue)	
		Mouse	Rat
0.0	7	5.27 ±0.79	5.69 ±0.52
0.1	7	5.80 ±0.50	6.99 ±0.86
1.0	7	5.73 ±0.46	6.25 ±0.58
10.0	7	5.83 ±0.44	5.51 ±1.38
0.0	14	6.73 ±1.02	5.28 ±0.84
0.1	14	11.55 ±2.83*	4.71 ±0.60
1.0	14	12.44 ±1.07*	5.07 ±0.73
10.0	14	11.11 ±0.38*	4.75 ±0.36
0.0	28	7.45 ±0.90	4.69 ±0.50
0.1	28	7.92 ±0.61	6.20 ±0.75
1.0	28	7.75 ±0.65	5.16 ±0.49
10.0	28	7.58 ±0.45	3.77 ±0.89
0.0	90	8.23 ±1.78	5.59 ±0.43
0.1	90	8.13 ±0.92	5.12 ±1.00
1.0	90	7.73 ±0.81	5.41 ±0.89
10.0	90	7.16 ±1.78	5.97 ±0.56

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc "t" test. * indicates value significantly different from that of control (no dieldrin).

Table 30: 7, 14, 28 and 90 Day Hepatic MDA Concentrations for B6C3F1 Mice and F344 Rats in Subchronic Dieldrin Study

Dieldrin (mg/kg diet)	Sampling Time (days)	Hepatic MDA (nmole/g tissue)	
		Mouse	Rat
0.0	7	14.50 ±5.93	11.37 ±4.44
0.1	7	29.70 ±7.92*	15.77 ±3.92
1.0	7	39.76 ±12.98*	11.99 ±5.08
10.0	7	45.00 ±23.38*	11.41 ±3.84
0.0	14	25.00 ±8.03	12.94 ±3.70
0.1	14	54.00 ±32.10	11.69 ±2.11
1.0	14	47.00 ±17.42	11.11 ±2.30
10.0	14	83.60 ±23.30*	11.81 ±1.99
0.0	28	55.00 ±37.77	11.37 ±4.44
0.1	28	39.40 ±13.39	15.77 ±3.92
1.0	28	48.80 ±12.03	11.99 ±5.08
10.0	28	38.50 ±6.07	11.41 ±3.84
0.0	90	30.80 ±3.83	11.65 ±4.40
0.1	90	37.40 ±8.11	9.12 ±3.51
1.0	90	31.80 ±9.36	9.96 ±1.18
10.0	90	36.20 ±9.91	8.64 ±3.69

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc "t" test. * indicates value significantly different from that of control (no dieldrin).

Table 31: 7, 14, 28 and 90 Day Urinary MDA Concentrations for B6C3F1 Mice and F344 Rats in Subchronic Dieldrin Study

Dieldrin (mg/kg diet)	Sampling Time (days)	Urine MDA (nmole/mg creatinine)	
		Mouse	Rat
0.0	7	87.93 ±9.89	72.60 ± 17.24
0.1	7	274.58 ±37.97*	70.20 ± 25.27
1.0	7	320.52 ±65.49*	79.60 ± 26.97
10.0	7	297.60 ±93.0*	54.60 ± 18.51
0.0	14	134.67 ±45.76	63.75 ± 3.77
0.1	14	268.33 ±31.09*	55.00 ± 13.29
1.0	14	234.33 ±41.40*	49.50 ± 7.00
10.0	14	251.00 ±76.22*	40.50 ± 15.42
0.0	28	126.67 ±27.54	99.00 ± 9.09
0.1	28	320.00 ±78.08*	72.25 ± 25.25
1.0	28	362.33 ±51.50*	71.75 ± 12.45
10.0	28	249.33 ±21.73*	88.75 ± 7.27
0.0	90	90.63 ±50.02	82.25 ± 19.31
0.1	90	187.78 ±100.73*	161.00 ± 28.55*
1.0	90	177.74 ±107.85*	307.75 ±105.05*
10.0	90	244.54 ±65.71*	739.00 ±105.19*

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc "t" test. * indicates value significantly different from that of control (no dieldrin).

Table 32: 7, 14, 28 and 90 Day Hepatic 8-hydroxy-2'-deoxyguanosine Concentrations for B6C3F1 Mice and F344 Rats in Subchronic Dieldrin Study

Dieldrin (mg/kg diet)	Sampling Time (days)	Hepatic oh8dG (oh8dG/dGuo x 10^{-5})	
		Mouse	Rat
0.0	7	5.00 ±0.80	3.04 ±1.42
0.1	7	6.28 ±1.32	1.96 ±0.87
1.0	7	4.42 ±0.82	3.80 ±0.92
10.0	7	6.01 ±0.71	3.59 ±1.15
0.0	14	4.36 ±1.32	3.77 ±1.02
0.1	14	3.26 ±1.18	3.38 ±0.50
1.0	14	2.89 ±1.04	3.69 ±0.51
10.0	14	3.18 ±1.64	3.35 ±1.13
0.0	28	4.26 ±0.72	3.94 ±0.71
0.1	28	5.36 ±0.78	2.55 ±0.66
1.0	28	5.43 ±1.52	3.46 ±1.26
10.0	28	3.38 ±1.76	2.79 ±0.87
0.0	90	5.44 ±2.01	2.85 ±0.54
0.1	90	5.59 ±0.83	3.29 ±0.67
1.0	90	4.97 ±1.16	3.02 ±0.66
10.0	90	5.70 ±0.74	2.50 ±0.66

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc "t" test. * indicates value significantly different from that of control (no dieldrin).

Table 33: 7, 14, 28 and 90 Day Urinary 8-hydroxy-2'-deoxyguanosine Concentrations for B6C3F1 Mice and F344 Rats in Subchronic Dieldrin Study

Dieldrin (mg/kg diet)	Sampling Time (days)	Urine oh8dG (nmole/mg creatinine)	
		Mouse	Rat
0.0	7	2.80 ±0.76	3.90 ± 0.63
0.1	7	2.84 ±0.42	2.87 ± 0.25
1.0	7	2.77 ±0.48	3.30 ± 1.19
10.0	7	2.49 ±0.30	2.06 ± 0.18
0.0	14	2.82 ±0.91	6.06 ± 1.39
0.1	14	4.36 ±0.17	8.94
1.0	14	5.68 ±1.95	4.84 ± 0.25
10.0	14	11.05 ±1.27*	8.88 ± 3.20
0.0	28	2.54	4.57 ± 0.74
0.1	28	3.02 ±1.61	4.11 ± 0.38
1.0	28	9.46	1.82 ± 0.01
10.0	28	10.18	1.86 ± 0.71
0.0	90	2.05	3.87 ± 0.81
0.1	90	2.07	5.09
1.0	90	1.92	2.84 ± 0.22
10.0	90	2.83	5.28 ± 0.94

Pooled urine samples were used for analysis. One to three samples were available per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc "t" test. * indicates value significantly different from that of control (no dieldrin). No standard deviations were shown if only one pooled sample was tested.

Aim 4: Determine if the oxidative stress induced by dieldrin in the B6C3F1 mouse can be modulated by dietary Vit E.

In the previous section, it was determined that dieldrin or ROS generated by dieldrin, modulated the antioxidant defense system of mouse and rat livers thus effecting the way this xenobiotic acts on these two species. Here, these parameters were again examined but multiple doses of dietary Vit E were employed in an attempt to modulate dieldrin's effect in mice. Specifically, dieldrin was administered p.o. to B6C3F1 mice and F344 rats by being mixed with NIH-07 diets and formulated into pellets by a commercial supplier (Dyets, Inc. Bethlehem, PA). Eight dose groups were used: 0, 50, 250, or 450 mg Vit E/kg diet (Kappius and Diplock, 1992) and 0, 50, 250, or 450 mg Vit E/kg diet with 10 mg dieldrin/kg diet (based on Walker, et al. 1972). 5 animals were used per dose group. Animals were sacrificed on days 14 and 28 days based on *in vivo* DNA synthesis studies conducted by Stevenson, et al. (1995). At time of sacrifice urine was removed from the bladder and blood was sampled from inferior vena cava. Livers were removed and rinsed in cool phosphate buffer saline solution. Livers were then sectioned into small slices weighing approximately 100 mg and stored in separate containers. All samples were immediately placed in liquid nitrogen and then stored at -80°C until used. Results were tabulated in three formats to facilitate analysis: 1) Vit E dose response only with no dieldrin, 2) paired

groups +/- dieldrin at a single Vit E dose and 3) the dieldrin groups with varying amounts of Vit E.

Body weights, liver weights and %LW/BW ratios are shown in tables 34, 35 and 36. The 0 mg Vit E/kg diet group showed a significant decrease in liver weigh and %LW/BW at both 14 and 28 days. While the 250 mg Vit E/kg diet also showed a decrease in %LW/BW at 14 days this was not observed at 28 days. All dieldrin treated mice showed a significant increase in liver weight and %LW/BW when compared to their corresponding nondieldrin treated counterparts at both 14 and 28 days. The body weight of mice tended to increase with dietary Vit E. This showed a statistical difference at day 28 with the 450 mg Vit E/kg group being higher than control. While the 0 mg Vit E/kg group initially lost weight, this effect was not observed at 28 days.

Molecular Antioxidant Status

Hepatic and serum Vit E are shown in tables 37, 38 and 39. Hepatic Vit E in the nondieldrin treated groups showed a positive correlation with dietary Vit E ($r^2 = 0.908$, $P<0.001$at 14 days; $r^2 = 0.942$ $P<0.001$ at 28 days). All groups, at both time points, were significantly different from the 50 mg Vit E/kg diet control. Likewise, hepatic Vit E in the 10 mg dieldrin/kg diet treated groups showed a positive correlation with dietary Vit E ($r^2 = 0.961$, $P<0.001$ at 14 days; $r^2 = 0.942$ $P<0.001$ at 28 days). Again, all groups at both

time points were significantly different from both the 50 mg Vit E/kg diet. Table 39 illustrates that dieldrin significantly reduced hepatic Vit E in all Vit E dose groups at both 14 and 28 days. Serum Vit E in the nondieldrin treated groups showed a positive correlation with dietary Vit E ($r^2 = 0.437$, $P<0.001$at 14 days; $r^2 = 0.851$ $P<0.001$ at 28 days). In contrast to the hepatic Vit E from dieldrin treated mice, only the 0 mg Vit E/kg diet group was significantly different from the 50 mg Vit E/kg diet group in the nondieldrin treated mice. At 28 days, all nondieldrin treated mice had significantly different serum Vit E when compared to the 50 mg Vit E/kg diet group. Likewise, serum Vit E in the 10 mg dieldrin/kg diet treated groups showed a positive correlation with dietary Vit E ($r^2 = 0.660$, $P<0.001$ at 14 days; $r^2 = 0.688$, $P<0.001$ at 28 days). Unlike hepatic Vit E, not all of the nondieldrin groups were significantly different from their dieldrin counterparts. In table 38, the 0 mg Vit E/kg diet group showed no significant difference at either 14 or 28 days. In addition, at 28 days only the 450 mg Vit E/kg diet groups showed a significant difference between the dieldrin and nondieldrin treated mice in serum Vit E.

Hepatic UA is shown in tables 40, 41 and 42. Hepatic UA showed only slight variation. At 14 days, a negative correlation was observed between dietary Vit E and hepatic UA ($r^2=0.762$, $P<0.001$) in the nondieldrin treated groups (Table 40). At 14 days, the 250 and 450 mg Vit E/kg diet were significantly decreased from the 50 mg Vit E/kg diet control. By day 28, this

decrease was no longer noticeable. However, the 0 mg Vit E/kg diet group did show a significant increase over the 50 mg Vit E/kg diet control. The dieldrin treated mice also showed a negative correlation with dietary Vit E at 14 days (r^2=0.5111, P<0.004) with the 0 mg Vit E/kg plus dieldrin and the 450 mg Vit E/kg plus dieldrin being significantly different from the 50 mg Vit E/kg plus dieldrin group (Table 41). By day 28, this effect was no longer apparent. The only significant difference between dieldrin and nondieldrin treated groups, at a given level of dietary Vit E, occurred on day 14 with the 0 mg Vit E/kg diet group.

Hepatic Vit C is shown in tables 40, 41 and 42. No significant difference existed between the nondieldrin treated Vit E groups at either day 14 or 28. This was also true for the dieldrin treated groups at day 14. At day 28, however, the 250 mg Vit E/kg diet group with dieldrin was significantly increased above the 50 mg Vit E/kg diet group with dieldrin. While the 450 mg Vit E/kg was not significantly elevated above the corresponding 50 mg Vit E/kg plus dieldrin group. A trend toward increasing Vit C with dietary Vit E in the dieldrin treated mice was still observed. Interestingly, all dieldrin treated mice at both 14 and 28 days had significantly elevated Vit C in contrast to their corresponding nondieldrin treated counterparts, as can be seen in table 42.

Hepatic GSH is also shown in tables 40, 41 and 42. Interestingly, both the nondieldrin treated mice (r^2=0.551, P<0.001) and the dieldrin treated

mice (r^2=0.771, P<0.001) showed an inverse correlation with dietary Vit E at day 14 but not at day 28. Interestingly, in the nondieldrin treated groups, the 250 and 450 mg Vit E/kg diet group were significantly below control (50 mg Vit E/kg diet) at day 14 but at day 28 these two groups were significantly above control. For the dieldrin treated mice, only the 450 mg Vit E/kg diet group was decreased from the 50 mg Vit E/kg diet group at day 14. Likewise, at day 28 in the dieldrin treated mice, only the 250 mg Vit E/kg diet group was significantly increased above control. The only significant difference observed between the nondieldrin and dieldrin treated mice at a given dietary Vit E concentration occurred at the 0 and 50 mg Vit E/kg diet. At 14 days, both the 0 and 50 mg Vit E/kg diet groups with dieldrin had significantly lower hepatic GSH than their corresponding nondieldrin treated group. At 28 days, however, a recovery had occurred and now the dieldrin treated mice in the 0 mg Vit E/kg diet group had higher levels than the nondieldrin treated group.

Oxidative Stress Status

Hepatic and urinary MDA and hepatic oh8dG were used to determine oxidative stress. MDA and oh8dG concentrations are shown in tables 43, 44 and 45. While hepatic oh8dG was slightly higher in the 0 mg Vit E/mg diet groups, this was only significantly increased above control (50 mg Vit E/kg diet) at 28 days in the nondieldrin treated mice. No difference was observed

between nondieldrin and dieldrin treated mice at a constant dietary Vit E level. This observation was consistent with the previous subchonic study in which mice on various doses of dieldrin showed no change in hepatic oh8dG. MDA in both urine and liver did show interesting trends. Both hepatic and urinary MDA in the nondieldrin treated 0 mg Vit E/kg diet group were significantly elevated above control at both days 14 and 28. Hepatic MDA in dieldrin treated mice showed a good negative correlation with dietary Vit E at both 14 and 28 days (r^2=0.734, P<0.0001; r^2=0.589, P<0.0001 respectively). Urinary MDA did not show this trend at 14 days, but at 28 days a negative correlation to dietary Vit E did exist among dieldrin treated mice (r2=0.660, P<0.0013). The intergroup variation between the nondieldrin and dieldrin treated mice was only noticeable at 0 and 50 mg Vit E/kg diet. At these dietary levels of Vit E, urinary MDA in the dieldrin treated mice was significantly above their nondieldrin treated counterparts at day 14. At day 28, only the 50 mg Vit E/kg diet group showed a significant increase in urinary MDA with dieldrin treatment. A similar pattern existed for the hepatic MDA in the 0 and 50 mg Vit E/kg diet group with one exception. On day 28, the 0 mg Vit E/kg diet group with dieldrin had a significantly lower level of hepatic MDA than the nondieldrin treated group.

Hepatic MDA varied inversely with hepatic Vit E levels in the dieldrin treated groups (r^2 = 0.982, P<0.001, r^2 = 0.586 P<0.001 at 14 days with and without dieldrin respectively) (figure 5). The decrease at later time points

may be a result of the higher levels of hepatic Vit C. Urinary MDA, while not organ specific, is useful to non evasively monitor the status of the rodents during the course of the study. Mouse urinary MDA from dieldrin treated animals was higher than control at all time points when contrasted to their nondieldrin counterparts at all doses of dietary Vit E (figure 6). Increased dietary Vit E tended to reduce urinary MDA by day 28. Hepatic oh8dG/dGuo did not vary significantly with addition of dieldrin to the diet in any dietary Vit E dose group. However, the 0 mg Vit E/kg diet group did have higher concentrations of oh8dG/dGuo than the other dietary Vit E groups. The 0 mg Vit E/kg diet group had significantly higher hepatic oh8dG/dGuo than the control (50 mg Vit E/kg diet). In summary, dieldrin-induced oxidative damage in mice could be modulated by dietary Vit E.

Table 34: 14 and 28 Day Subchronic Dieldrin Study with Dietary Vit E Supplementation in B6C3F1 Mice: Body Weights, Liver Weights and Percent Liver Weight/Body Weight for 0, 50, 250 and 450 mg Vit E/kg Diet

Vit E Dose (mg/kg diet)	Sampling time (days)	BW (grams)	LW (grams)	LW/BW x 100
0	14	24.19 ±1.80*	1.20 ±0.07*	4.97 ±0.16*
50	14	26.45 ±1.92	1.43 ±0.13	5.42 ±0.26
250	14	27.04 ±1.73	1.34 ±0.07	4.96 ±0.25*
450	14	26.72 ±0.99	1.44 ±0.11	5.38 ±0.26
0	28	26.88 ±0.56	1.22 ±0.07*	4.54 ±0.16*
50	28	26.68 ±0.76	1.52 ±0.09	5.69 ±0.28
250	28	28.34 ±1.40	1.60 ±0.10	5.63 ±0.25
450	28	28.94 ±1.32*	1.61 ±0.17	5.54 ±0.37

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc Fisher's Protected Least Significant Difference. * indicates value significantly different from that of control (50 mg Vit E/kg diet without dieldrin).

Table 35: 14 and 28 Day Subchronic Dieldrin Study with Dietary Vit E Supplementation in B6C3F1 Mice: Body Weights, Liver Weights and Percent Liver Weight/Body Weight for 0, 50, 250 and 450 mg Vit E/kg Diet with 10 mg Dieldrin/kg diet

Vit E Dose (mg/kg diet)	Dieldrin Dose (mg/kg diet)	Sampling time (days)	BW (grams)	LW (grams)	LW/BW x 100
50		14	26.45 ±1.92	1.43 ±0.13	5.42 ±0.26
0	10	14	24.39 ±1.04	1.36 ±0.09a,b	5.57 ±0.43a,b
50	10	14	25.64 ±1.86	1.70 ±0.11a	6.65 ±0.30a
250	10	14	25.00 ±1.04	1.64 ±0.09a	6.57 ±0.20a
450	10	14	26.34 ±1.95	1.61 ±0.15a	6.11 ±0.18a,b
50		28	26.68 ±0.76	1.52 ±0.09	5.69 ±0.28
0	10	28	26.80 ±1.66	1.66 ±0.27a	5.91 ±0.65a,b
50	10	28	27.27 ±1.87	1.90 ±0.13a	6.97 ±0.31a
250	10	28	27.79 ±1.45	2.06 ±0.20a	7.40 ±0.45a
450	10	28	26.55 ±1.05	1.82 ±0.05a	6.87 ±0.18a

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc Fisher's Protected Least Significant Difference. 'a' indicates value significantly different from that of control (50 mg Vit E/kg diet without dieldrin) and 'b' indicates value significantly different from that of 50 mg Vit E/kg diet with 10 mg dieldrin/kg diet.

Table 36: 14 and 28 Day Subchronic Dieldrin Study with Dietary Vit E Supplementation in B6C3F1 Mice: Body Weights, Liver Weights and Percent Liver Weight/Body Weight for 0, 50, 250 and 450 mg Vit E/kg Diet with and without 10 mg Dieldrin/kg diet

Vit E Dose (mg/kg diet)	Dieldrin Dose (mg/kg diet)	Sampling time (days)	BW (grams)	LW (grams)	LW/BW x 100
0		14	24.19 ±1.80	1.20 ±0.07	4.97 ±0.16
0	10	14	24.39 ±1.04	1.36 ±0.09*	5.57 ±0.43*
50		14	26.45 ±1.92	1.43 ±0.13	5.42 ±0.26
50	10	14	25.64 ±1.86	1.70 ±0.11*	6.65 ±0.30*
250		14	27.04 ±1.73	1.34 ±0.07	4.96 ±0.25
250	10	14	25.00 ±1.04	1.64 ±0.09*	6.57 ±0.20*
450		14	26.72 ±0.99	1.44 ±0.11	5.38 ±0.26
450	10	14	26.34 ±1.95	1.61 ±0.15*	6.11 ±0.18*
0		28	26.88 ±0.56	1.22 ±0.07	4.54 ±0.16
0	10	28	26.80 ±1.66	1.66 ±0.27*	5.91 ±0.65*
50		28	26.68 ±0.76	1.52 ±0.09	5.69 ±0.28
50	10	28	27.27 ±1.87	1.90 ±0.13*	6.97 ±0.31*
250		28	28.34 ±1.40	1.60 ±0.10	5.63 ±0.25
250	10	28	27.79 ±1.45	2.06 ±0.20*	7.40 ±0.45*
450		28	28.94 ±1.32	1.61 ±0.17	5.54 ±0.37
450	10	28	26.55 ±1.05	1.82 ±0.05*	6.87 ±0.18*

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc Fisher's Protected Least Significant Difference. * indicates value significantly different from that of corresponding nondieldrin treated group.

Table 37: 14 and 28 Day Subchronic Dieldrin Study with Dietary Vit E Supplementation in B6C3F1 Mice: Hepatic and Serum Vit E Concentrations for 0, 50, 250 and 450 mg Vit E/kg Diet

Vit E Dose (mg/kg diet)	Sampling time (days)	Hepatic Vit E (μmoles/g tissue)	Serum VE (μmoles/ml serum)
0	14	0.54 ±0.03*	0.33 ±0.05*
50	14	1.10 ±0.09	0.64 ±0.10
250	14	3.33 ±0.77*	0.67 ±0.08
450	14	4.70 ±0.78*	0.72 ±0.09
0	28	0.61 ±0.12*	0.19 ±0.02*
50	28	1.19 ±0.18	0.74 ±0.07
250	28	3.43 ±0.52*	1.38 ±0.20*
450	28	5.90 ±0.98*	1.76 ±0.29*

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc Fisher's Protected Least Significant Difference. * indicates value significantly different from that of control (50 mg Vit E/kg diet without dieldrin).

Table 38: 14 and 28 Day Subchronic Dieldrin Study with Dietary Vit E Supplementation in B6C3F1 Mice: Hepatic and Serum Vit E Concentrations for 0, 50, 250 and 450 mg Vit E/kg Diet with 10 mg Dieldrin/kg diet

Vit E Dose (mg/kg diet)	Dieldrin Dose (mg/kg diet)	Sampling time (days)	Hepatic Vit E (µmoles/g tissue)	Serum VE (µmoles/ml serum)
50		14	1.10 ±0.09	0.64 ±0.10
0	10	14	0.14 ±0.03a,b	0.32 ±0.10a
50	10	14	0.77 ±0.13a	0.35 ±0.04a
250	10	14	2.03 ±0.18a,b	0.46 ±0.05a
450	10	14	3.02 ±0.31a,b	0.53 ±0.06a,b
50		28	1.19 ±0.18	0.74 ±0.07
0	10	28	0.45 ±0.05a,b	0.17 ±0.04a,b
50	10	28	0.91 ±0.08a	0.61 ±0.04a
250	10	28	1.95 ±0.30a,b	1.23 ±0.08a,b
450	10	28	3.03 ±0.43a,b	1.10 ±0.08a,b

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc Fisher's Protected Least Significant Difference. 'a' indicates value significantly different from that of control (50 mg Vit E/kg diet without dieldrin) and 'b' indicates value significantly different from that of 50 mg Vit E/kg diet with 10 mg dieldrin/kg diet.

Table 39: 14 and 28 Day Subchronic Dieldrin Study with Dietary Vit E Supplementation in B6C3F1 Mice: Hepatic and Serum Vit E Concentrations for 0, 50, 250 and 450 mg Vit E/kg Diet with and without 10 mg Dieldrin/kg diet

Vit E Dose (mg/kg diet)	Dieldrin Dose (mg/kg diet)	Sampling time (days)	Hepatic Vit E (µmoles/g tissue)	Serum VE (µmoles/ml serum)
0		14	0.54 ±0.03	0.33 ±0.05
0	10	14	0.14 ±0.03*	0.32 ±0.10
50		14	1.10 ±0.09	0.64 ±0.10
50	10	14	0.77 ±0.13*	0.35 ±0.04*
250		14	3.33 ±0.77	0.67 ±0.08
250	10	14	2.03 ±0.18*	0.46 ±0.05*
450		14	4.70 ±0.78	0.72 ±0.09
450	10	14	3.02 ±0.31*	0.53 ±0.06*
0		28	0.61 ±0.12	0.19 ±0.02
0	10	28	0.45 ±0.05	0.17 ±0.04
50		28	1.19 ±0.18	0.74 ±0.07
50	10	28	0.91 ±0.08	0.61 ±0.04
250		28	3.43 ±0.52	1.38 ±0.20
250	10	28	1.95 ±0.30*	1.23 ±0.08
450		28	5.90 ±0.98	1.76 ±0.29
450	10	28	3.03 ±0.43*	1.10 ±0.08*

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc Fisher's Protected Least Significant Difference. * indicates value significantly different from that of corresponding nondieldrin treated group.

Table 40: 14 and 28 Day Subchronic Dieldrin Study with Dietary Vit E Supplementation in B6C3F1 Mice: Hepatic UA, Vit C and GSH Concentrations for 0, 50, 250 and 450 mg Vit E/kg Diet

Vit E Dose (mg/kg diet)	Sampling time (days)	Hepatic UA (μmoles/g tissue)	Hepatic Vit C (μmoles/g tissue)	Hepatic GSH (μmoles/g tissue)
0	14	8.72 ±0.48	0.88 ±0.07	8.33 ±0.83
50	14	8.85 ±0.44	0.98 ±0.06	8.00 ±0.24
250	14	7.77 ±1.34*	0.87 ±0.04	6.26 ±1.29*
450	14	6.87 ±0.50*	1.02 ±0.11	5.10 ±0.43*
0	28	9.45 ±0.49*	1.31 ±0.09	3.51 ±0.45*
50	28	8.17 ±0.36	0.91 ±0.07	6.55 ±0.73
250	28	8.07 ±0.92	0.94 ±0.11	7.80 ±1.19*
450	28	8.27 ±0.75	1.45 ±0.14	8.82 ±0.49*

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc Fisher's Protected Least Significant Difference. * indicates value significantly different from that of control (50 mg Vit E/kg diet without dieldrin).

Table 41: 14 and 28 Day Subchronic Dieldrin Study with Dietary Vit E Supplementation in B6C3F1 Mice: Hepatic UA, Vit C and GSH Concentrations for 0, 50, 250 and 450 mg Vit E/kg Diet with 10 mg Dieldrin/kg diet

Vit E Dose (mg/kg diet)	Dieldrin Dose (mg/kg diet)	Sampling time (days)	Hepatic UA (μmoles/g tissue)	Hepatic Vit C (μmoles/g tissue)	Hepatic GSH (μmoles/g tissue)
50		14	8.85±0.44	0.98±0.06	8.00±0.24
0	10	14	9.97±0.51a,b	2.28±0.08a	7.03±0.41a
50	10	14	8.48±0.50	2.33±0.16a	6.52±1.18a
250	10	14	7.78±1.10	2.49±0.44a	5.80±1.31a
450	10	14	7.21±0.25b	2.64±0.14a	4.68±0.58a,b
50		28	8.17±0.36	0.91±0.07	6.55±0.73
0	10	28	9.52±1.51	3.84±0.94a	5.13±0.67a
50	10	28	8.18±0.56	2.98±0.22a	6.73±0.55
250	10	28	8.54±0.86	3.83±0.34a,b	8.92±1.00b
450	10	28	8.57±1.61	4.77±1.01a	7.88±1.68

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc Fisher's Protected Least Significant Difference. 'a' indicates value significantly different from that of control (50 mg Vit E/kg diet without dieldrin) and 'b' indicates value significantly different from that of 50 mg Vit E/kg diet with 10 mg dieldrin/kg diet.

Table 42: 14 and 28 Day Subchronic Dieldrin Study with Dietary Vit E Supplementation in B6C3F1 Mice: Hepatic UA, Vit C and GSH Concentrations for 0, 50, 250 and 450 mg Vit E/kg Diet with and without 10 mg Dieldrin/kg diet

Vit E Dose (mg/kg diet)	Dieldrin Dose (mg/kg diet)	Sampling time (days)	Hepatic UA (µmoles/g tissue)	Hepatic Vit C (µmoles/g tissue)	Hepatic GSH (µmoles/g tissue)
0		14	8.72 ±0.48	0.88 ±0.07	8.33 ±0.83
0	10	14	9.97 ±0.51*	2.28 ±0.08*	7.03 ±0.41*
50		14	8.85 ±0.44	0.98 ±0.06	8.00 ±0.24
50	10	14	8.48 ±0.50	2.33 ±0.16*	6.52 ±1.18*
250		14	7.77 ±1.34	0.87 ±0.04	6.26 ±1.29
250	10	14	7.78 ±1.10	2.49 ±0.44*	5.80 ±1.31
450		14	6.87 ±0.50	1.02 ±0.11	5.10 ±0.43
450	10	14	7.21 ±0.25	2.64 ±0.14*	4.68 ±0.58
0		28	9.45 ±0.49	1.31 ±0.09	3.51 ±0.45
0	10	28	9.52 ±1.51	3.84 ±0.94*	5.13 ±0.67*
50		28	8.17 ±0.36	0.91 ±0.07	6.55 ±0.73
50	10	28	8.18 ±0.56	2.98 ±0.22*	6.73 ±0.55
250		28	8.07 ±0.92	0.94 ±0.11	7.80 ±1.19
250	10	28	8.54 ±0.86	3.83 ±0.34*	8.92 ±1.00
450		28	8.27 ±0.75	1.45 ±0.14	8.82 ±0.49
450	10	28	8.57 ±1.61	4.77 ±1.01*	7.88 ±1.68

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc Fisher's Protected Least Significant Difference. * indicates value significantly different from that of corresponding nondieldrin treated group.

Table 43: 14 and 28 Day Subchronic Dieldrin Study with Dietary Vit E Supplementation in B6C3F1 Mice: Hepatic MDA, Hepatic 8-hydroxy-2'-deoxyguanosine and Urinary MDA Concentrations for 0, 50, 250 and 450 mg Vit E/kg Diet

Vit E Dose (mg/kg diet)	Sampling time (days)	Hepatic MDA (nmole/g tissue)	Urine MDA (nmole/mg creatinine)	Hepatic oh8dG (oh8dG/dGuo x 10^{-5})
0	14	38.87 ±12.48*	315.06 ±52.07*	4.58 ±0.74
50	14	16.60 ±2.56	114.92 ±41.31	4.02 ±0.98
250	14	19.64 ±3.41	143.41 ±12.71	3.99 ±0.88
450	14	10.04 ±2.09	170.76 ±32.71	3.56 ±0.24
0	28	108.98 ±16.20*	409.96 ±18.99*	4.68 ±0.87*
50	28	24.94 ±2.75	99.46 ±19.34	3.43 ±0.69
250	28	24.26 ±6.39	69.29 ±11.45	3.90 ±0.69
450	28	13.60 ±2.32*	78.68 ±17.87	3.60 ±1.06

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc Fisher's Protected Least Significant Difference. * indicates value significantly different from that of control (50 mg Vit E/kg diet without dieldrin).

Table 44: 14 and 28 Day Subchronic Dieldrin Study with Dietary Vit E Supplementation in B6C3F1 Mice: Hepatic MDA, Hepatic 8-hydroxy-2'-deoxyguanosine and Urinary MDA Concentrations for 0, 50, 250 and 450 mg Vit E/kg Diet with 10 mg Dieldrin/kg diet

Vit E Dose (mg/kg diet)	Dieldrin Dose (mg/kg diet)	Sampling time (days)	Hepatic MDA (nmole/g tissue)	Urine MDA (nmole/mg creatinine)	Hepatic oh8dG (oh8dG/dGuo x 10^{-5})
50		14	16.60 ±2.56	114.92 ±41.31	4.02 ±0.98
0	10	14	69.15 ±5.48a,b	235.77 ±3.44a,b	4.90 ±0.31
50	10	14	33.99 ±4.41a	372.38 ±70.91a	3.85 ±1.36
250	10	14	22.70 ±4.15b	195.47 ±29.47b	3.18 ±0.53
450	10	14	9.65 ±2.17b	235.16 ±50.26b	3.49 ±0.94
50		28	24.94 ±2.75	99.46 ±19.34	3.43 ±0.69
0	10	28	73.48 ±20.25a,b	403.12 ±50.71a,b	4.61 ±1.06
50	10	28	38.57 ±8.41a	232.83 ±80.26a	3.54 ±1.15
250	10	28	28.63 ±4.53	100.73 ±3.18b	3.84 ±0.80
450	10	28	17.01 ±5.09b	96.38 ±21.34b	3.54 ±0.73

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc Fisher's Protected Least Significant Difference. 'a' indicates value significantly different from that of control (50 mg Vit E/kg diet without dieldrin) and 'b' indicates value significantly different from that of 50 mg Vit E/kg diet with 10 mg dieldrin/kg diet.

Table 45: 14 and 28 Day Subchronic Dieldrin Study with Dietary Vit E Supplementation in B6C3F1 Mice: Hepatic MDA, Hepatic 8-hydroxy-2'-deoxyguanosine and Urinary MDA Concentrations for 0, 50, 250 and 450 mg Vit E/kg Diet with and without 10 mg Dieldrin/kg diet

Vit E Dose (mg/kg diet)	Dieldrin Dose (mg/kg diet)	Sampling time (days)	Hepatic MDA (nmole/g tissue)	Urine MDA (nmole/mg creatinine)	Hepatic oh8dG (oh8dG/dGuo x 10^{-5})
0		14	38.87 ±12.48	315.06 ±52.07	4.58 ±0.74
0	10	14	69.15 ±5.48*	235.77 ±3.44*	4.90 ±0.31
50		14	16.60 ±2.56	114.92 ±41.31	4.02 ±0.98
50	10	14	33.99 ±4.41*	372.38 ±70.91*	3.85 ±1.36
250		14	19.64 ±3.41	143.41 ±12.71	3.99 ±0.88
250	10	14	22.70 ±4.15	195.47 ±29.47	3.18 ±0.53
450		14	10.04 ±2.09	170.76 ±32.71	3.56 ±0.24
450	10	14	9.65 ±2.17	235.16 ±50.26	3.49 ±0.94
0		28	108.98 ±16.20	409.96 ±18.99	4.68 ±0.87
0	10	28	73.48 ±20.25*	403.12 ±50.71	4.61 ±1.06
50		28	24.94 ±2.75	99.46 ±19.34	3.43 ±0.69
50	10	28	38.57 ±8.41*	232.83 ±80.26*	3.54 ±1.15
250		28	24.26 ±6.39	69.29 ±11.45	3.90 ±0.69
250	10	28	28.63 ±4.53	100.73 ±3.18	3.84 ±0.80
450		28	13.60 ±2.32	78.68 ±17.87	3.60 ±1.06
450	10	28	17.01 ±5.09	96.38 ±21.34	3.54 ±0.73

Five animals were used per dose group per time period. Results are expressed as mean ± standard deviation. Statistical analysis was accomplished using ANOVA ($P<0.05$ criteria) followed by the post hoc Fisher's Protected Least Significant Difference. * indicates value significantly different from that of corresponding nondieldrin treated group.

DISCUSSION

A. SPECIES COMPARISON

The basal levels of oxidative stress among mice and rats showed a strong correlation with the susceptibility of species and strains to hepatic carcinogenesis. ROS are believed to play an important role in carcinogenesis, especially promotion (Trush and Kensler, 1991). oh8dG is an important marker of oxidative damage. The higher hepatic oh8dG in some animal species or strains might reflect the combined effects of: 1) higher metabolite rate, 2) a lower antioxidant defense system, or 3) a lower activity of DNA repair (Trush and Kenseler, 1991; Breimer, 1990; Floyd, 1990). This higher concentration of oh8dG might than suggest higher risk to chemical carcinogenesis (Floyd, 1990). Hepatic oh8dG decreased in the C3H/He, B6C3F1 and C57BL/6 mouse and this decrease correlated with their decreased susceptibility to carcinogenesis (r^2=0.996, $p<0.05$). According to Drinkwater and Bennett (1991), estimation of relative susceptibility are 50% for the C3H/He mouse, 25% for the B6C3F1 mouse and 1% for the C57BL/6 mouse. The C3H/He mouse has a relative susceptibility to hepatocarcinogenesis fifty times greater than the C57BL/6 mouse. It also had twice as much oh8dG than the C57BL/6 mouse. The B6C3F1 was intermediate between its parents. Furthermore, hepatic oh8dG in rat livers was lower than in mouse livers which agreed with the higher incidence of hepatocarcinogenesis in mice.

Whether oxidative stress is in itself sufficient to cause oxidation of DNA or whether the oxidized DNA is genomic or mitochondria are two other questions to consider. Hypomethylation of DNA has been associated with carcinogenesis among mice strains. Ray and coworkers (1994) showed that the B6C3F1 mouse is less capable of maintaining methylation of the c-raf oncogene than is the more resistance C57Bl. This site is not even methylated in the C3H parental strain which is the most susceptible to cancer. This methylation of DNA may protect it against oh8dG formation caused by oxidative stress. When the hydroxyl radical reacts with DNA it forms an intermediate DNA radical on the nitrogen of guanosine. This is the same site which is methylated. Work by Nicotera (1991) supports this hypothesis. Nicotera studied the effects of antioxidants on the formation of sister chromatid exchange in cells from Bloom's syndrome patients. Bloom's syndrome is an autosomal recessive disorder characterized by high incidence of cancer at an early age. Both hypomethylation and an increase in oxidative stress have been implicated in this disorder. His results showed a decrease in sister chromatid exchange with an increase in antioxidants, suggesting oxidative stress may be critical to this process. In addition, Hinrichsen and coworkers (1990) fed rats a choline deficient diet which produces hypomethylation and observed a large increase of oh8dG in the liver. These studies then suggest that not only is the antioxidant/pro-oxidant balance important in carcinogenesis but the hypomethylation of genomic DNA may

be a prerequisite which allows the increase ROS formation access to DNA binding sites.

Another question to consider is whether the oh8dG damage occurs in genomic DNA or mitochondrial DNA. Cattley and Glover (1993) showed that while clofibric acid increased oh8dG in whole cell DNA extracts, no increase was observed when only nuclear extracts were examined. This suggest that the oxidative damage caused by this peroxisome proliferator was a result of oxidation of mitochondrial DNA. Interestingly, Shay and Werbin (1992) suggested that mutations in mtDNA may result in initiated cells. These changes might then give these cells a selective growth advantage allowing for clonal expansion (promotion). In this study, total DNA was examined for oxidative damage. Mitochondrial DNA represents only 1% of cellular DNA. A significant change in mitochondrial DNA damage may not be possible to determine when examing total cellular DNA as was done in this study.

No major trends existed among the enzymatic antioxidants and basal hepatic oh8dG concentrations. The C3H mouse, which is most susceptible to spontaneous tumor formation, had the highest catalase activity suggesting that this is not a pivotal enzyme relating to tumor formation. The other enzymes showed no constant trends between mouse strains and rat and spontaneous tumor formation suggesting that they are not major factors in the carcinogenesis process.

On the other hand, molecular antioxidants did differ between mice and rats. Small molecular antioxidants, such as GSH, Vit C, Vit E and UA, have been shown to play an important role in the defense against oxidative damage and are involved in increasing the life span of various species (Bocker et al., 1993; NiKi et al., 1991; Kaplowitz and Ookhtens, 1985). Therefore, high hepatic oh8dG might be indicative of low antioxidant concentrations. Interestingly, only a weak correlation between oh8dG and GSH was found between the livers of mouse interstrains; and surprisingly, low oh8dG correlated with low GSH concentraions in rat liver. However, the higher Vit C and Vit E in rat livers do correlate with the low oh8dG suggesting that these non-enzymatic antioxidants may play a major role in the regulation of oh8dG.

The major role of GSH in radical scavenging reactions is reducing hydrogen peroxide produced by liver mitochondria. Its steady-state cellular concentration is achieved by a balance between the synthesis rate and utilization rate (Kaplowitz and Ookhtens, 1985). The different concentrations of hepatic GSH between micee and rats and among mouse interstrains suggest different enzyme activity or constituted pattern of enzyme system, which is controlled by genetics. As was found with other investigators (Neal et al., 1987; Monroe and Eaton 1987), the values obtained for GST were much higher in mouse strains than in the rat while only a slight difference could be observed in GSH-Px activity. GSH is a substrate

for: 1) GST, which functions in detoxification via conjugation, and 2) GSH peroxidase, which functions to reduce peroxides (Kaplowitz and Ookhtens 1985). The higher hepatic GSH in mice may be more a function of the GST pathway than the antioxidant defense systems. Parke and Ioannides (1990) suggest that the high rate of GST activity in the mice may result in a depletion of GSH during detoxification reactions leaving the mouse more suceptible to oxidative stress. This is supported by other researchers who suggest the GST conjugation pathway correlates with risk of cancer in mice (Green, 1990; Vos and Bladeren, 1990).

In general, this species comparison linked basic oxidative stress with animal susceptibility to carcinogenesis. It suggest the possible effects of Vit E and Vit C in protection against DNA oxidative damage by free radicals in animal organs. From the data presented, it appears that the mouse has a higher basal level of oxidative stress than the rat. The mouse also has lower concentrations of critical antioxidants such as Vit E and Vit C.

The species difference observed here has been the subject of several investigations. Parke and Ioannides (1990) contend that the mouse due to its smaller size and higher metabolism may exist in a more stressed condition. This is in part supported by work by Sohal et al., (1990) and Ku et al., (1993) who compared mitochondria activity among several species. In this system, the mouse produced more oh8dG than the rat. This then agrees with Sohal

and coworkers who found higher superoxide production in mouse mitochondria then in the rat.

B. REACTIVE OXYGEN SPECIES GENERATION

The reaction of ROS with salicylate followed by HPLC analysis of the 2,3-DHBA adduct has been adapted in this study for the detection of ROS in microsomes and primary hepatocytes. Salicylate can form several adducts on exposure to ROS. These include catechol, 2,3-DHBA and 2,5-DHBA. Of these three compounds only catechol and 2,3-DHBA are exclusively produced by reaction with the hydroxyl radical. The other common adduct, 2,5-DHBA, can be generated by direct metabolism of salicylic acid via the P-450 system. The 2,5-DHBA increased in both rat and mouse in all incidences of dieldrin dosing. A change in the concentration of the 2,5-DHBA might reflect either competition for the P-450 isoenzyme metabolizing salicylate or a possible increase in its metabolism by the interaction of another substance with the cytochrome P-450 macromolecule. Since the 2,3-DHBA did not increase in either rat microsomes or hepatocytes, it suggest that dieldrin may have synergistically participated in the metabolism of salicylic acid to 2,5-DHBA. Dieldrin may have caused a conformational change in the P-450 isoenzyme that metabolizes salicylic acid and enhanced salicylic acid metabolism. The fact that mouse microsomes produce free radicals supports previous work in which GJIC communication by dieldrin could be reversed by the antioxidant

Vit E in dieldrin treated mouse hepatocytes (Bachowski et al., 1995). This role of oxidative stress is further supported by the reduction of DNA S-phase synthesis in B6C3F1 mice by dieldrin when supplementing the diet with Vit E, a potent free radical scavenger (Stevenson et al., 1995).

Parke and Ioannides state that the mouse can engage in futile cycling in which a compound can continually cycle through the cytochrome P-450 system and generate ROS. They suggest that compounds can have an affinity for the P450 system but are metabolically oxygenated with difficulty. Thus, the activated oxygen of the P-450 is released as superoxide and not inserted into the xenobiotic substrate. This results in futile cycling. They contend that the P-450 IIB system which can use aldrin, the parent compound of dieldrin, as a substrate can engage in futile cycling in this matter. The P-450 IIE system (ethanol metabolism) and the P-450 IV (peroxisome proliferator metabolism) can also generate free radicals in this fashion. Thus, the generation of free radicals through activation of the P-450 system may be a common route of tumorgenicity for many structurally unrelated compounds. In fact, Guengerich (1991) suggests that the cytochrome P-450 system is critical to the carcinogenic process.

In a multi-species study by Wright et al., (1972), dieldrin-induced alterations in subcellular structure were reversible in the rat, mouse, beagle dog and rhesus monkey. In all three species, microsomal protein activity increased and an increase in smooth endoplasmic reticulum was observed.

The rhesus monkey used in this study showed only a slight increase in microsomal protein activity with no increase in liver size. An increase in tumor incidences in these species was only observed in mice (Walker et al., 1969). Work by Baldwin & Robinson (1972) and later Hutson (1976) showed that the mouse was slower to metabolize dieldrin and that it did not excrete the pentachloroketone metabolite which predominated in the rat. Interestingly, the mouse did produce several polar metabolites not seen in the rat. When Obenholser (1977) blocked the P-450 metabolisms of dieldrin in the rat using SKF-525A new metabolites were reportedly formed. When these facts are considered in light of the selective production of free radicals in mice, it suggest that a difference in the way each of these species metabolize dieldrin may contribute to dieldrin's selective carcinogenic effect in mice.

C. DIELDRIN ALTERATIONS IN THE ANTIOXIDANT/PRO-OXIDANT BALANCE

Many xenobiotics have been associated with a pertubation of this antioxidant/pro-oxidant balance. Paraquat and CCl_4 increased free radicals and decrease GJIC in rat hepatocytes (Ruch & Klaunig, 1988). Phenobarbital & linoleate have been shown to decrease Vit E in the male F344/N rat (Hendrich, 1991). DDT induced lipid peroxidation in the male Wistar rat (Barros, 1994). TCDD resulted in mitochondrial lipid peroxidation in female Sprague-Dawley rats (Stohs et al., 1990). Nafenopin, a peroxisome proliferator, increases conjugated dienes in F344 rat hepatocytes (Tomaszewski et al., 1990). These xenobiotics may induce oxidative stress in cells by direct formation of free radicals, altering enzymatic systems (such as cytochrome P-450, peroxisomes or mitochondria) which generate free radicals, decreasing the antioxidant defense system or over running the repair mechanisms.

Antioxidant / Pro-oxidant Balance

In the previous sections, the mouse was shown to have a naturally higher pro-oxidant level than the rat as evidenced by higher basal hepatic oh8dG concentrations. This higher pro-oxidant state is further impacted by the production of ROS by dieldrin treatment of mouse microsomes and hepatocytes. The increase in ROS observed in mouse hepatocytes correlated

with the decrease in hepatocyte Vit E (r^2= 0.80), the increase in hepatocyte MDA (r^2=0.76) and the increase in hepatocyte oh8dG (r^2=0.51). This suggest that oxidative stress in mouse hepatocytes results from both a decrease in the antioxidant balance (Vit E) and an increase in the pro-oxidant balance (ROS generation). Surprisingly, the increase in Vit C and GSH were either not sufficient to protect the hepatocytes or occurred secondary to the initial decrease in Vit E and resulting oxidative stress. Results from the 90 day mouse vs. rat subchronic study suggest that the latter may be true since in that study the increase in Vit C appears to quench the oxidative stress in the liver.

The antioxidants measured in this 90 day study, Vit E, Vit C, UA and GSH, represent the major non enzymatic antioxidants in the liver. At early time points, a dose-dependent decrease in hepatic Vit E was observed in mice with dieldrin treatment which correlated with the observed increase in MDA (r^2 = 0.954; $P<0.05$ at day 7 and r^2 = 0.804; $P<0.05$ at day 14). Later (days 28 and 90), the dose response could not be observed possibly due to bioaccumulation of dieldrin in the liver, an increase in other hepatic antioxidants or improved elimination of hepatic MDA. Interestingly, GSH initially increased but later returned to basal levels in mice. This may be due to the dramatic increase in hepatic Vit C. In fact, Vit E, Vit C and β-carotene have been shown to depress GSH synthesis (Sohal et al., 1985). It

appears than, that the initial drop in Vit E led to a burst of GSH synthesis but later when Vit C increased this effect was diminished.

A similar correlation of Vit E and MDA was observed when dietary Vit E was used to modulated dieldrin's hepatotoxic effects. In this study, a strong negative correlation also existed between MDA and Vit E in the dieldrin treated mice ($r^2 = 0.84$; $P<0.05$ at 14 days and $r^2 = 0.78$; $P<0.05$ at 28 days). In the nondieldrin treated mice, the effect of dietary Vit E on the interaction of MDA and hepatic Vit E, while not as strong, was still present ($r^2 = 0.56$; $P<0.05$ at 14 days and $r^2 = 0.46$; $P<0.05$ at 28 days). Likewise, table 46 shows that both MDA and Vit E were strongly influenced by dietary Vit E. The decrease in Vit E with dieldrin treatment may be due to its displacement from the membrane, oxidation by free radicals or interference with its transport in the body. Since the rat does not show an increase in ROS, the other two choices appear more likely.

It is interesting to note that while mouse hepatocytes showed an increase in oh8dG, mouse livers did not except in mice fed a Vit E deficient diet. This may reflect the inability of primary cultured hepatocytes to repair DNA damage or that in vitro doses of dieldrin used may not accurately reflect in vivo concentrations.

Oxidative Stress in Relation to DNA S-phase Synthesis

During the 90 day subchronic dieldrin study, DNA S-phase synthesis initially rose but later returned to near basal levels. Figure 38 examines this phenomena in light of the antioxidant/pro-oxidant balance. The increase in DNA S-phase synthesis at 10 mg dieldrin/kg diet coincided with an increase in urinary oh8dG and hepatic MDA. By day 28, both oxidative stress markers and DNA S-phase synthesis were decreasing. Interestingly, this coincided with an increase in hepatic Vit C. This might suggest that a critical redox level exist in the liver above which oxidative stress occurs and in turn triggers DNA S-phase synthesis. In support of this argument, table 47 shows the Pearson correlation (r) for antioxidants and oxidative stress markers as related to DNA S-phase synthesis. As can be seen at early time points, both hepatic and serum Vit E have a strong negative correlation with DNA synthesis while hepatic MDA has a strong positive correlation. At later times, when oxidative stress was no longer observed these correlations were not significant.

The induction of DNA synthesis in subchronic rodent studies is a predictive tool to evaluate the carcinogenicity of xenobiotics (Busser and Lutz, 1987). Other investigators have reviewed the connection between cell proliferation and cancer (Butterworth and Goldsworthy, 1982 and Melnick, 1992). The mouse vs rat subchronic dieldrin study related DNA synthesis to oxidative stress. When cells are in S-phase they are more vulnerable to the

genotoxic effects of chemicals since the time for DNA repair is limited and the DNA is more exposed (Cunningham and Matthews, 1991). Indeed, oh8dG concentration increased in mouse urine in relation to the increase in DNA S-phase synthesis suggesting that the DNA was more exposed to the effects of oxidative stress at this time. However, it is also more likely to be repaired at this time. Since hepatic oh8dG did not change, it appears that most of the oxidative damage was repaired. An alternative hypothesis could include the damage occurring at a site other than the liver. This stimulation of DNA synthesis prior to formation of preneoplastic foci may allow for the fixation of spontaneously occurring mutations or mutations resulting from genotoxic compounds. This would explain the tumor occurrences observed by dieldrin in the absence of an initiating agent.

Oxidative stress may cause an increase in DNA S-phase synthesis through a variety of mechanisms: 1) Vitamin E has been shown to inhibit PKC activity which is involved in many cellular proliferation pathways (Mahoney et al., 1988). Furthermore, work by Charpentier and coworkers (1993) suggest that the increase in hepatic and serum Vit E resulting from increase dietary intake increases secretion of TGF-β, an antiproliferative agent. The decrease in hepatic Vit E observed in this study then might explain the proliferative event. 2) Activation of oncogenes such as AP-1 or NF-kB by oxidative stress (Sorta et al., 1990; Schreck et al., 1992) might result in an increase in DNA synthesis. In support of this argument,

activation of AP-1 has been associated with increasing GST activity (Storta et al., 1990). Interestingly, dieldrin also has been reported to induce GST (Moody et al., 1991), suggesting a possible connection between dieldrin's selective hepatic action in mice and AP-1 induction. 3) Loss of cellular homeostasis may lead to a proliferative event. The increase of MDA in mouse livers on dieldrin diet is indicative of membrane lipid peroxidation. Changes in cellular membranes can result in a large variety of functional alterations within the cell which may lead to cellular proliferation or tumor promotion (Williams, 1981; Klaunig ,1991). Oxidative stress can induce changes in cellular homeostasis such as alteration in calcium (Swann et al., 1991 and Allen et al., 1992), intracellular pH (Masaki et al., 1989; Gores et al., 1989) and inhibition of gap junction intercellular communication (GJIC) (Ruch et al., 1991). Release of calcium ions from cellular stores may in turn lead to mitotic events such as activation of the calcium dependent PKC (Kretsinger et al., 1980). Likewise, many tumor-promoting agents exhibit the ability to disrupt and inhibit GJIC (Trosko and Chang, 1984; Klaunig et al., 1991). It has been previously shown that the B6C3F1 mouse hepatocyte GJIC was inhibited by dieldrin treatment whereas the F344 rat hepatocytes are not. Furthermore, this inhibition was reversed by addition of Vit E to the media. In contrast, rat hepatocytes exhibited no decrease in GJIC following exposure to dieldrin.. Ruch and Klaunig have shown paraquat-generated oxygen free radicals to inhibit gap junction intercellular communication. This

may suggest that inhibition of GJIC in mouse hepatocytes is a result of lipid peroxidation following dieldrin treatment.

Noninvasive Biomarkers of Oxidative Stress

Detection and monitoring of toxic events by noninvasive processes is critical for the application of rodent toxicological studies to the human. Urinary oh8dG is an excellent biomarker of oxidative stress since it can not be absorbed through the gastro-intestinal tract nor is it formed enzymatically from dGuo in the body (Simic, 1994). In the mouse vs rat subchronic dieldrin study, urinary oh8dG parallel the induction of DNA S-phase synthesis (a classical endpoint of hepatic carcinogenesis studies). Likewise, in these studies another urinary marker of oxidative stress, MDA, was related back to its hepatic concentrations (tables 48 and 49). The correlation of urinary MDA to hepatic MDA had its strongest correlation at earlier time points in the mouse 90 day study (r^2 = 0.92, and 0.76 on days 7 and 14, respectively; $P<0.05$). Likewise, on the dietary Vit E study, good correlations were observed in the dieldrin groups between hepatic and urinary MDA study (r^2 = 0.985, and 0.948 on days 14 and 28, respectively; $P<0.05$). Thus, while not organ specific (unless prior knowledge of target organ is known), these two markers of oxidative stress offer good alternatives for toxicological analysis.

In a similar fashion, hepatic and serum Vit E also showed excellent correlations in both in vivo studies (tables 48 and 49). Showing correlations

of r2 > 0.94 for all time points in the 90 day mouse subchronic study. While not as dramatic, the dietary Vit E study also showed good correlation between hepatic and serum Vit E. Thus, like MDA and oh8dG, serum Vit E is an excellent indicator of systemic toxicity.

Figure 38: Examination of Influence Dieldrin-Induced Oxidative Stress on DNA S-Phase Synthesis in B6C3F1 Mouse Liver

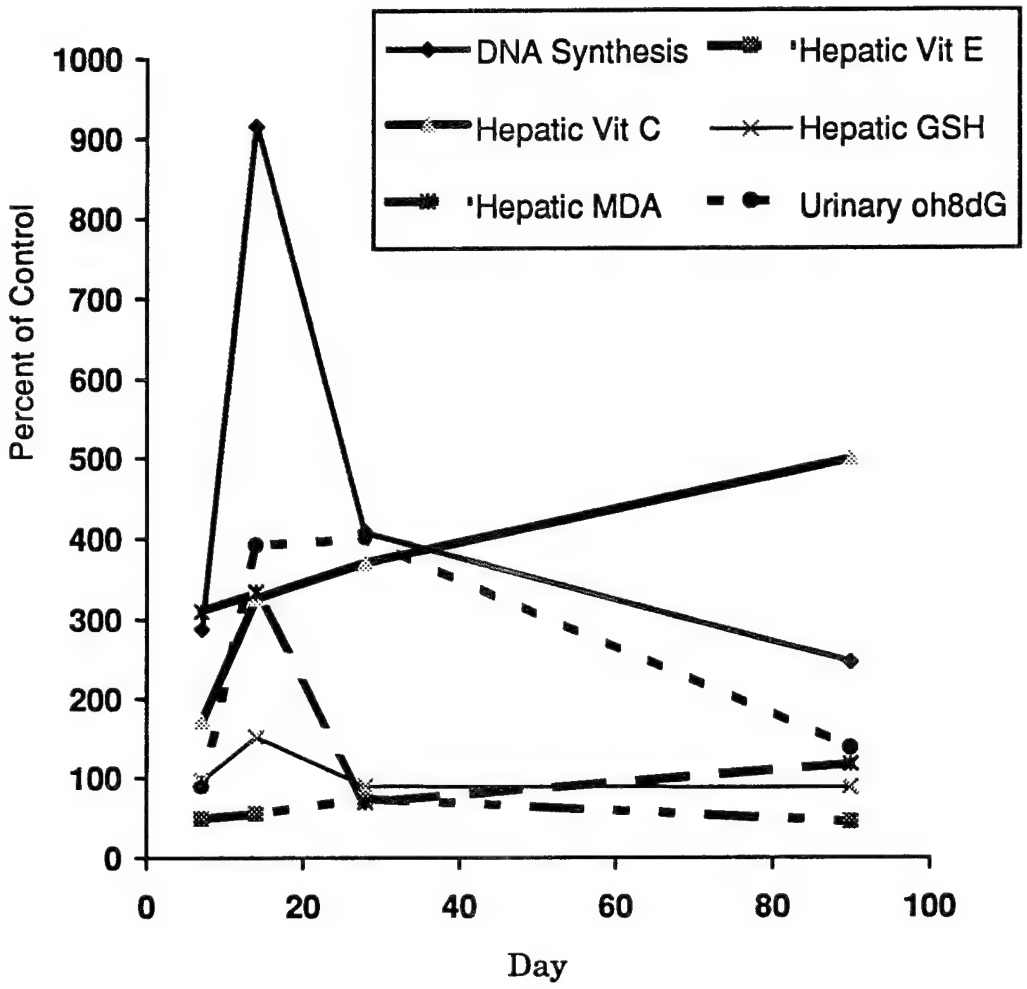


Figure 34: Correlation of antioxidant/pro-oxidant markers for 10 mg dieldrin/kg diet. All data is shown as percent of control at each time point. Urinary oh8dG concentrations followed the same pattern as the induction of DNA S-phase synthesis in mouse livers. GSH initially increased but later returned to control values. Interestingly, the decrease in DNA S-phase synthesis seems to occur when hepatic Vit C begins to climb. When the antioxidant level (Vit C in this case) rose sufficiently to reduce the pro-oxidant state of the liver, DNA S-phase synthesis was reduced.

Table 46: Correlation values for 28 Day B6C3F1 Mouse Dietary Vitamin E Subchronic Study showing Correlation of Dietary Vit E to Hepatic Vit E, Serum Vit E, Hepatic MDA and Urinary MDA

	Hepatic Vit E	Serum Vit E	Hepatic MDA	urine MDA
without dieldrin				
Day 14	0.987	0.546	0.558	0.148
Day 28	0.999	0.921	0.471	0.419
with dieldrin				
Day 14	0.983	0.987	0.755	0.202
Day 28	0.996	0.701	0.729	0.727

Table 47: Pearson's Coefficient for 90 Day B6C3F1 Mouse Subchronic Study for Days 7 and 14 Relating DNA S-phase Synthesis to Antioxidant/Pro-oxidant Balance

	Day 7	Day 14
Hepatic Vit E	-0.86	-0.67
Serum Vit E	-0.69	-0.65
Hepatic GSH	-0.63	0.30
Urinary MDA	0.49	0.38
Hepatic UA	0.50	0.26
Hepatic MDA	0.79	0.89
Hepatic Vit C	0.96	0.96
Urinary oh8dG	N.S.	0.97

N.S. indicates correlation was not significant ($P<0.05$)

Table 48: Correlation Analysis for 90 Day B6C3F1 Mouse Subchronic Study

Relating Hepatic Vit E vs Serum Vit E and Hepatic MDA vs. Urinary MDA

Days of Dieldrin Treatment	Hepatic Vit E vs Serum Vit E	Hepatic MDA vs Urinary MDA
7	0.962	0.921
14	0.999	0.760
28	0.967	N.S.
90	0.943	N.S.

N.S. indicates correlation was not significant ($P<0.05$)

Table 49: Correlation Analysis for 28 Day B6C3F1 Mouse Dietary Vitamine Subchronic Study Relating Hepatic Vit E vs Serum Vit E and Hepatic MDA vs. Urinary MDA

	Hepatic Vit E vs Serum Vit E	Hepatic MDA vs Urinary MDA
without dieldrin		
Day 14	0.587	0.740
Day 28	0.912	0.985
with dieldrin		
Day 14	0.996	N.S.
Day 28	0.739	0.948

N.S. indicates correlation was not significant ($P<0.05$)

Implication of Research to Carcinogenesis

Oxidative stress is a pervasive phenomenon resulting from the *in vitro* or *in vivo* treatment of hepatocytes with tumor promoting xenobiotics. These xenobiotics may act through a variety of difference mechanisms (i.e., peroxisome proliferation, direct production of free radicals by cytochrome P-450 system or by altering the antioxidant defense system of the cell). This researched focused on the selective action of dieldrin in mouse liver prior to the appearance of preneoplastic foci and tumors. The question arises as to how this oxidative stress observed with dieldrin treatment correlates to hepatocarcinogenicity observed in mice. Several possibilities exist which focus on nongenotoxic mechanisms of cancer: 1) the interrelationship between oxidative stress, aging and cancer, 2) redox regulation of transcription factors and other proteins or 3) a change in mitochondrial gene expression.

Oxidative Stress, Cancer and Aging

When van Ravenzwaay and coworkers (1988) examined the phenomena of nuclear polyploidization and tumor incidence in dieldrin treated CF-1 mice, they observed that liver tumor formation was associated with a constant level of polyploidization. Dieldrin accelerated the rate at which this polyploidization occurred. Since polyploidization is an age dependent process, they concluded that dieldrin may act by advancing the

biological age of CF-1 mouse livers (Shima and Sugahara, 1976). In another study, Ravenzwaay and colleagues (1988) examined changes in isoenzyme composition in the livers of CF-1 mice with dieldrin. They found that the expression of cytoplasmic A-alanine-aminotransferase isoenzyme decreased with age and that dieldrin accelerated this process. These two experiments led them to conclude that dieldrin operates by accelerating biological aging. The mechanism behind this process is not clear; however, the oxidative stress theory of aging is a major theme in gerontology (Harman 1968).

Several investigators have examined the role of antioxidants and aging. De and Darad (1991) studied the antioxidant concentrations in male Wistar rats at 3, 12 and 24 months of age. They found that serum concentrations of Vit E, Vit C and GSH were reduced by about 50% in the serum from 3 to 24 months. However, only GSH was significantly reduced in the liver. In a similar fashion, Vericel and coworkers (1994) found a decrease in both hepatic Vit E and GSH in Wistar-Kyoto rats and an increase in lipid peroxidation. Vit E concentrations at three months fell from 88 nmol/mg protein to 21 nmol/mg protein at 12 months and GSH concentrations fell from 3.4 nmoles/mg protein to 0.4 nmol/mg protein at 12 months. At the same MDA concentrations measured using the thiobarbituric assay increased four fold. In another study Vina and coworkers (1992) examined C57Bl mice and found the ratio of oxidized GSH to reduced GSH was double in older mice when compared to the younger ones. This implies

that the reduction in GSH seen with age is not due to its decrease synthesis but to an increased oxidative challenge. This increase oxidative challenge may arise from mitochondrial mutations or peroxisome dysfunctions occurring during the aging process (Bandy and Davison 1990; Masters and Crane 1995). Thus, there seems to be evidence for an increase in oxidative stress in the aging process.

The role of oxidative stress in carcinogenesis also has many supporters. Oxidative stress has been proposed as a mechanism by many investigators for carcinogenesis process (Trush and Kensler, 1984, Sun, 1990; Cerutti and Trump, 1991, Borek, 1991; Frenkel, 1992; Bankson, 1993; Clayson, 1993; Schwartz, 1993). It may then be possible that dieldrin's selective hepatocarcinogenicity in mice is a result of increasing the biological age of the liver by raising the basal level of oxidative stress in this target organ.

Redox modulation Cellular Components:

Another possible explanation for the hepatocarcinogenic action of dieldrin by oxidative stress may reside in a change in the redox state of the cell. Many cellular components have sites on them which are sensitive to changes in the antioxidant/pro-oxidant balance. Work by Baeuerle and Baltimore (1988) provided evidence for the release of the inhibitory IκB subunit from the NF-κB heterodimer in the presence of hydrogen peroxide.

Likewise, Schreck and coworkers showed that TNFα was a strong inducer of NF-κB. TNFα is a proven generator of ROS. Likewise, Abate and coworkers (1990) found that the DNA binding of Fos-Jun (AP-1) heterodimer was modulated by the reduction or oxidation of a single cysteine residue in the DNA binding domain of the two proteins. It seems quite possible than that the redox state of the cell may act as a second messenger system in the modulation transcription factors. Both of these transcription factors have been associated with cell proliferation.

Mitochondria DNA Alterations

DNA damage resulting from oxidative stress may be in either the genomic DNA or mitochondrial DNA (mtDNA). Richter (1992) reported mtDNA to have 16 times higher basal levels of oxidative damage then genomic in rat hepatocytes. This may be a result of increased production of free radical inside the mitochondria or a result of 'naked' DNA in the mitochondria. Furthermore, Shay & Werbin (1992) and Hadler (1989) reported that mtDNA fragments can be inserted into genomic DNA as a result of aging or oxidative damage; thus acting like an oncogenic virus. Bandy and Davison (1990) in a review article cited the ability of mictochondrial mutations to produce changes in cell surfaces whcih may allow descendence cells the ability to unify and enhance chances for survival and compete with normal cells for resources. This may in fact explain the

promotion ability of many xenobiotics. These changes in cell surfaces can likewise influence GJIC. An incease in oh8dG, as observed in mouse hepatocytes, may then not necessarily indicate a mutagenic event in genomic DNA but may rather be symptomatic of mitochondrial alterations.

CONCLUSIONS

The mouse exist in a naturally higher level of oxidative stress than the rat as evidenced by its higher basal level of hepatic oh8dG. This agrees with work by Sohal et al., (1990) in which he observed a higher production of ROS production in mouse mitochondria than in rat. Only, mouse microsomes and hepatocytes produced ROS following dieldrin treatment. This suggest a unique metabolic mechanism exist in mice but not rats with respect to dieldrin. Non-enzymatic antioxidants appear to reverse dieldrin's hepatic action in both in vivo studies either by an increase in Vit C as seen in the mouse vs rat subchronic study or by increasing dietary Vit E as seen in the dietary Vit E supplementation study.

The in vivo mouse and rat subchronic studies further showed a good correlation between the induction of oxidative stress and the occurrence of DNA S-phase synthesis suggesting this to be a critical event in dieldrin's action. This is further supported by the fact that at later time points DNA S-phase synthesis returned to basal level and the oxidative stress in the liver decreased in a similar fashion.

Thus, dieldrin's selective hepatic action in mice appears to result from the selective induction of oxidative stress in mouse livers.

BIBLIOGRAPHY

REFERENCES

Abate, C., Patel, L., Rauschen, R., Curran, T. (1990): Redox regulation of Fos and Jun DNA binding activity in vitro. *Science* **249**: 1157-1161.

Aebi, H. (1974): Catalase. In H.U. Bergmeyer (Ed.) Methods of Enzymatic Analysis. Academic Press. New York. pp673-684.

Allen, R.G. and Venkatraj, V.S. (1991): Oxidants and antioxidants in development and differentiation. Symposium: Regulation of Antioxidant Enzymes. American Institute of Nutrition. pp631-636.

Alsharif, N.Z., Lawson, T., and Stohs, S.J. (1994): 2,3,7,8-tetrachlorodibenzo-p-dioxin is mediated by the aryl hydrocarbon (Ah) receptor complex. *Toxicology*. **92(1-3):** 39-51.

Ames, B.N., Catheart, R., Schwiers, E., and Hochstein, P. (1981): Uric acid provides an antioxidant defense in humans against oxidant and radical caused aging and cancer: a hypothesis. *Proc. Natl. Acad Sci. USA* **78**: 6858-6862

Ames, B.N. and Gold, L.S. (1992): Animal cancer tests and cancer prevention. *Monographs-National Cancer Institute*. **12:** 125-32.

Andersen, H.R. and Andersen, O. (1993): Effects of dietary α-tocopherol and β-carotene on lipid peroxidation induced by methyl mercuric chloride in mice. *Pharmacology and Toxicology*. **73:** 192-201.

Arnaud, J., Blachier, F.S., Kia, D., and Favier, A. (1991): Simultaneous determination of retinol, alpha-tocopherol and beta-carotene in serum by isocratic high-performance liquid chromatography. *Journal of Chromatography*. **572:** 103-116.

Aust, S.D. and White, B.C. (1985): Iron chelation prevents tissue injury following ischemia. *Adv Free Rad Biol. Med*. **1:** 1-17.

Babior, B.M. (1984): Oxidants from phagocytes: agents of defense and destruction. *Blood*. **64(5):** 959-966.

Bachowski, S., Baker, T., Stevenson, D., Walborg, E., and Klaunig, J.E. (1995): The potential role of oxidative stress in nongenotoxic carcinogenesis in the mouse liver. In Slaga, Goldsworthy, Henry and Stevenson (Eds), In Progress in Clinical and Biological Research: Growth Factors and Tumor Promotion: Implications for Risk Assessment Conference, Barton Creek, TX. , Wily and Liss, NY. 1995.

Baeuerle, P.A. and Baltimore, D. (1988): IkB: a specific inhibitor of the NF-kB transcription factor. *Science.* **242:** 540-546.

Bagchi, D., Bagchi, M., Hassoun, E., Moser, J. and Stohs, S.J. (1993): Effects of carbon tetrachloride, menadione, and paraquat on the urinary excretion of malondialdehyde, formaldehyde, acetaldehyde, and acetone in rats. *Journal of Biochemical Toxicity.* **8(2):** 101-106.

Bagchi, M. and Stohs, S.J. (1993): In vitro induction of reactive oxygen species by 2,3,7,8-tetrachlorodibenzo-p-dioxin, endrin, and lindane in rat peritoneal macrophages and Hepatic Mitochondria and microsomes. *Free Radical Biology and Medicine.* **14:** 11-18.

Bagchi, M., Hassoun, E.A., Bagchi, D. and Stohs, S.J. (1993): Production of reactive oxygen species by peritoneal macrophages, hepatic mitochondria and microsomes from endrin-treated rats. *Free Radical Biology and Medicine.* **14:** 149-155.

Balansky, R.M., Blagoeva, P.M., Mircheva, Z.I., Stoitchev, I., and Chernozemski, I. (1986): The effect of antioxidants on MNNG-induced stomach carcinogenesis in rats. *Journal of Cancer Research and Clinical Oncology.* **112(3):** 272-275.

Baldwin, M.K. and Robinson, J. (1972): A comparison of the metabolism of HEOD (Dieldrin) in the CF1 mouse with that in the CFE rat. *Food and Cosmetic Toxicology.* **10:** 333-351.

Bandy, B. and Davison. A.J. (1990): Mitochondrial mutations may increase oxidative stress: Implications for carcinogenesis and aging? *Free Radical Biology and Medicine.* **8:** 523-539.

Bankson, D.D., Kestin, M. and Rifai, N. (1993): Role of free radicals in cancer and atherosclerosis. *Nutrition support.* **13(2):** 463-480.

Barros, S.B., Pimente, R., Simizu, K. Azzalis, L.A., Costa, I.S., and Junqueira, B.C. (1994): Dose-dependent study of liver lipid peroxidation

related parameters in rats treated with pp'-DDT. *Toxicology Letters.* **79**:33-38.

Bauer-Hofmann, R., Buchmann, A., Mahr, J., Kress, S. and Schwarz, M. (1992): The tumour promoters dieldrin and phenobarbital increase the frequency of c-Ha-ras wild-type, but not of c-Ha-ras mutated focal liver lesions in male C3H/He mice. *Carcinogenesis.* **13(3):** 477-481.

Becker, B.F. (1992): Towards the physiological function of uric acid. *Free Radical Biology and Medicine.* **14:** 615-631.

Bellomo, G., Mirabelli, F., Richelmi, P., and Orrenius, S. (1983): Critical role of sulfhydryl groups(s) in the ATP-dependent Ca^{2+} sequestration by the plasma membrane fraction from rat liver. *FEBS Letter.* **163:** 136-139.

Bendich A., Mechin, L.J. and Scandura, O. (1986): The antioxidant role of Vitamin C. *Ach Free Radical Bil Med.* **2**: 419-444.

Benson, A.M. Batzinger, R.P. Ou, S-Y.L., Buedling, E., Cha, Y-N. and Talalay, P. (1978): Elevation of hepatic glutathione S-transferase activities and protection against mutagenic metabolites of benzo(a)pyrene by dietary antioxidants. *Cancer Research.* **38**: 4486-4495.

Bevenue, A., Hylin, J.W., Kawano, Y., Kelly, T.W., (1972): Organochlorine pesticides residues in water, sediment, algae, and fish: Hawaii, 1970-1971. *Pest. Monit. J.* **6:** 56-64.

Bergelson, S., Pinkus, R. and Daniel, V. (1994): Intracellular glutathione levels regulate Fos/Jun induction and activation of glutathione S-transferase gene expression. *Cancer Research* **54**: 36-41.

Bocker, B.F. (1993): Towards the physiological function of uric acid. *Free Radical Biol. & Med.* **14**: 615-631.

Boelsterli, U.A., Wolf, A. and Goldlin, C. (1993): Oxygen free radical production mediated by cocaine and its ethanol-derived metabolite, cocaethylene, in rat hepatocytes. *Hepatology.* **18(5):** 1154-1161.

Borek, C. (1991): Free-radical processes in multistage carcinogenesis. *Free Radical Research Communications.* **12/13**: 745-750.

Boveris, A., Oshino, N., and Chance, B. (1972): The cellular production of hydrogen peroxide. *Biochem J.* **128**: 617-630.

Breimer, L.H. (1990): Molecular mechanisms of oxygen radical carcinogenesis and mutagenesis: The role of DNA base damage. *Molecular Carcinogenesis.* **3**:188-197.

Brown, R.K. and Kelly, F.J. (1994): Evidence for increased oxidative damage in patients with cystic fibrosis. *Pediatric Research.* **36(4):** 487-493.

Burke, M.D., Thompson, S., Elcombe, C.R., Halpert, J., Haaparanta,T., and Mayer,R.T. (1985): Ethoxy-, pentoxy-, and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. *Biochemical Pharmacology.* **34(18):** 337-3345.

Busser, M-T. and Lutz, W.K. (1987): Stimulation of DNA synthesis in rat and mouse liver by various tumor promoters. *Carcinogenesis.* **8(10):** 1433-1437.

Butterworth, B.E. and Goldsworthy, T.L. (1991): The role of cell proliferation in multistage carcinogenesis. *Cell Proliferation.* **198(2):** 683-687.

Cameron, T.P., Hickman, R.L., Kornreich, M.R. and Tarone, R.E. (1985): History, survival, and growth patterns of B6C3F1 mice and F344 rats in the National Cancer Institute carcinogenesis testing program. *Fundamenetal and Applied Toxicology.* **5:** 526-538.

Carfagna, M.A., Held, S.D., and Kedderis, G.L. (1993): Furan-induced cytolethality in isolated rat hepatocytes: correspondence with in vivo dosimetry. *Toxicology and Applied Pharmacology.* **123(2):** 265-73.

Cattley, R.C. and Glover, S.E. (1993): Elevated 8-hydroxydeoxyguanosine in hepatic DNA of rats following exposure to peroxisome proliferators: Relationship to carcinogenesis and nuclear localization. *Carcinogenesis.* **14(12)**: 2495-2499.

Cerutti, P.A. (1991): Oxidant stress and carcinogenesis. *European Journal of Clinical Investigation.* **21**: 1-5.

Cerutti, P.A. and Trump, B.F. (1991): Inflammation and oxidative stress in carcinogenesis. *Cancer Cells.* **3(1)**: 1-7.

Chang, T.C., Chang, M.J. and Hsueh, S. (1993): Glutathione concentration and distribution in cervical cancers and adjacent normal tissues. *Gynecologic & Obstetric Investigation.* **36(1):** 52-55

Chaudhary, A.K., Nokubo,M., Marnett,L.J. and Blair, I.A. (1994): Analysis of the Malondialdehyde-2'-deoxyguanosine Adduct in Rat Liver DNA by Gas

Chromatography/Electron Capture Negative Chemical ionization Mass Spectrometry. *Biological Mass Spectrometry*. **23:** 457-464.

Charpentier, A., Groves, S., Simmons-Menchaca, M., Turley, J., Zhao, B., Sanders, B.G. and Kline, K. (1993): RRR-α-tocopheryl succinate inhibits proliferation and enhances secretion of transforming growth factor β (TGF-β) by human breast cancer cells. *Nutrition and Cancer*. **19(3):** 225-239.

Chen, L.H. (1989): Interaction of vitamin E and ascorbic acid (Review). *In Vitro*. **3**: 199-210.

Chen, L.H., Thacker, R.R., and Chow, C.K. (1980): Tissue anitoxidant status and related enzymes in rats with long-term vitamin E deficiency *Nutrition Reports International*. **22(6):** 873-879.

Chen, Y.Y. (1992): Effects of benzene on lipid peroxidation and the activity of relevant enzymes in humans. *Chinese Journal of Preventive Medicine*. **26(6):** 336-338.

Chung F-L, and Xu,Y. (1992): Increased 8-oxydeoxyguanosine levels in lung DNA of A/J mice and F344 rats treated with the tobacco-specific nitrosamine 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone. *Carcinogenesis*. **13**:1269-1272.

Church, W.H. and Ward. V. L. (1994): Uric acid is reduced in the substantia nigra in Parkinson's disease: effect on dopamine oxidation. *Brain Research Bulletin*. **33(4):** 419-423.

Clayson, D.B., Mehta, R., and Iverson, F. (1994): Oxidative DNA damage--The effects of certain genotoxic and operationally non-genotoxic carcinogens. *Mutation Research*. **317**: 25-42.

Cohen, G., Dembiec, D., and Marcus, J. (1970): Measurement of catalase activity in tissue extracts. *Analytical Biochemistry*. **34**: 30-38.

Columbano, A., Ledda-Columbano, G.M., Ennas, M.G., Curto, M., Chelo, A. and Pani, P. (1987): Inability of mitogen-induced liver hyperplasia to support the induction of enzyme-altered islands induced by liver carcinogens. *Cancer Res.* **47**: 5557-5559.

Columbano, A., Ledda-Columbano, G.M., Coni, P., Pichiri-Coni, G., Curto, M., and Pani, P. (1991): Chemically induced cell proliferation and carcinogenesis: differential effect of compensatory cell proliferation and

mitogen-induced direct hyperpplasia on hepatocarcinogenesis in the rat. *Progress in Clinical & Biological Research*. **369:** 217-225.

Cunningham, M.L. and Matthews, H.B. (1991): Relationship of hepatocarcinogenicity and hepatocelluar proliferation induced by mutagenic noncarcinogens vs carcinogens. *Toxicology and Applied Pharmacology*. **110:** 505-513.

Curley, A. and Kimbrough, R. (1969): Chlorinated hydrocarbon insecticides in plasma and milk of pregnant and lactating women. *Arch. Envirom. Hlth.* **18:** 156-164.

Cutler, R.G. (1984): Antioxidants, aging, and longevity. In: Pryor, W.A., ed. *Free Radicals in Biology*. Vol. VI. Orlando: Academic Press: 371-428.

Davies, K.J.A. (1986): Intracellular proteolytic systems may function as secondary antioxidants defenses: an hypothesis. *Journal of Free Radical Biology and Medicine*. **9:** 155-173.

Davies, K.J.A., Sevanian, A., Muakkassah-Kelly, S.F., Hochstein, P. (1986): Uric acid-iron ion complexes. *Biochem. Journal.* **235:** 747-754.

Davis, K.J. and Fitzhugh, O.G. (1962): tumorigenic potential of aldrin and dieldrin for mice. *Toxic. Appl. Pharmac.* **4:** 187-189.

De, A.K. and Darad, R. (1991): Age-associated changes in antioxidants and antioxidative enzymes in rats. *Mechanisms of Aging and Development*. **59:** 123-128.

De Duve, C. (1983): Microbodies in the living cell. *Sci. Am.* **248**: 74-84.
de Jong, G. (1991) Long-term health effects of aldrin and dieldrin. *Toxicology Letters Suppl.* Amsterdam: Elsevier Science Publishers B.V., pp 1-206.

Dees, C. and Travis, C. (1993): The mitogenic potential of trichloroethylene in B6C3F1 mice. *Toxicology Letters*. **69:** 129-137.

Deichmann, W.B., MacDonald, W.E., Blum, E. Bevilacqua, M. Radomski,J., Keplinger, M. and Balkus, M. (1970): Tumorigenicity of aldrin, dieldrin and endrin in the albino rat. *Industrial Medicine and Surgery*. **39(10):** 426-434.

Decker, K. (1993): Mechanisms and mediators in hepatic necrosis. *Gastroenterologia Japonica*. **28(suppl 4):** 21-35.

Deubert, K.H. and Zuckerman, B.M. (1969): Distribution of dieldrin and DDT in cranberry bog soil. *Pest. Monit. J.* **2**: 172-175.

Devary, Y., Gottlieb, R.A., Lau, L.F. and Karin, M. (1991): Rapid and preferential activation of the c-jun gene during the mammalian UV response. *Molecular and Cellular Biology*. **11:** 2804-2811.

Diwan, B.A., Ward, J.M. and Rice, J.M. (1991): Modification of liver tumor development in rodents. In: Ito, N. and Sugano, H. (eds) Modification of Tumor Development in Rodents. *Prog. Exp. Tumors Res.* Basel, Karger. **33:** 76-107.

Drinkwater, N.R. and Bennett, L.M. (1991): Genetic control of carcinogenesis in experimental animals. In: Ito, N. and Sugano, H. (eds): Modification of tumor development in rodents. *Prog. Exp. Tumor Res.* Basel, Karger. **33:** 1-20

Ehrmann,R.L. and Gey,G.O. (1956): The growth of cells on a transparent gel of reconstituted rat-tail collagen. *Journal of the National Cancer Institute.* **16(6):** 1375-1389.

Eldridge, S.R., Goldsworthy, T.L., Popp, J.A., and Butterworth, B.E. (1992): Mitogenic stimulation of hepatocellular proliferation in rodents following 1,4-dichlorobenzene administration. *Carcinogenesis.* **12:** 1557-1561.

Eldridge, S.R., Tilbury, L.F., Goldsworthy, T.L. and Butterworth, B.E. (1990): Measurement of chemically induced cell proliferation in rodent liver and kidney: a comparison of 5-bromo-2'-deoxyuridine and [3H]thymidine administered by injection or osmotic pump. *Carcinogenesis.* **11(12)**: 2245-2251.

ElSisi, A.E.D., Earnest, D.L. and Sipes, I.G. (1992): Vitamin A potentiation of carbon tetrachloride hepatotoxicity: role of liver macrophages and active oxygen species. *Toxicology and applied Pharmacology*. **119**: 295-301.

Evans, V.G. (1993): Multiple pathways to apoptosis. *Cell Biology International.* **17(5):** 461-476.

Floyd, R.A. (1990): The role of 8-hydroxyguanine in carcinogenesis. *Carcinogenesis.* **11(9):** 1447-1450.

Floyd, R.A., Watson, J.J. and Wong, P.K. (1984): Sensitive assay of hydroxyl free radical fornmation utilizing high pressre liquid chromatography with

electrochemical detection of phenol and salicylate hydroxylation products. *Journal of Biochemical and Biophysical Methods*. **10**: 221-235.

Floyd, R.A., West, M.S., Eneff, K.L., Schneider, J.E., Wong, P.K., Tingey, D.T., and Hogsett, W.E. (1990): Conditions influencing yield and analysis of 8-hydroxy-2'-deoxyguanosine in oxidatively damage DNA. *Analytical Biuocheistry*. **188**: 155-158.

Freeman, B.A. and Crapo, J.D. (1982): Biology of Disease: Free radicals and tissue injury. *Laboratory Investigation*. **47(5):** 412-426.

Frenkel, K. (1991): Carcinogen-mediated oxidant formation and oxidative DNA damage. *Pharmac. Ther.* **53**: 127-166.

Gad, S.C. and Weil, C.S. (1989): Chapter 15 Statistics for toxicologist. procedures and methods of toxicology. Raven Press, Ltd.

Gerber, M., Richardson, S., Favier, F., and Crastes de Paulet, A. (1990): Vitamin E and tumor growth. In: Emerit et al. Ed. Antioxidants in Therapy and Preventive Medicine. New York. Plenum Press.

Gerrish, K.E. and Gensler, H.L. (1993): Prevention of photocarcinogenesis by dietary vitamin E. *Nutrition and Cancer*. **19(2):** 125-133.

Glauert, H.P., Beaty, M.M., Clark, T.D., Greenwell, W.S., Tatum, V., Chen, L.C., Borges, T., Clark, T.L., Srinivasan, S.R., and Chow, C.K. (1990): Effect of dietary vitamin E on the development of altered hepatic foci and hepatic tumors induced by the peroxisome proliferator ciprofibrate. *Journal of Cancer Research and Clinical Oncology*. **116(4):** 351-356.

Goel, M.R., Shara, M.A. and Stohs, S.J.(1988): Induction of lipid peroxidation by hexachlorocyclohexane, dieldrin, TCDD, carbon tetrachloride, and hexachlororbenzene in rats. *Bull. Environ. Contam. Toxicol.* **40:**255-262.

Green, T. (1990): Species differences in carcinogenicity: The role of metabolism in human risk evaluation. *Teratogenesis, Carcinogenesis, and Mutagenesis.* **10**: 103-113.

Grootveld, M. and Halliwell, B. (1986): Aromatic hydroxylation as a potential measure of hydroxyl-radical formation in vivo. *Biochem. J.* **237:** 499-504.

Guengerich, F.P. (1991): reactions and significance of cytochrome P-450 enzymes. *Journal of Biological Chemistry*. **266(16):** 10019-10022.

Guo, N., Conaway, C.C., Huyssain, N.S., and Fiala, E.S. (1990): Sex and organ differences in oxidative DNA and RNA damage due to treatment of Sprague-Dawley rats with acetoxime or 2-nitropropane. *Carcinogenesis.* **11(9):** 1659-1662.

Gut, A., Shiel, N., Kay, P.M., Segal, I., and Braganza, J.M. (1994): Heightened free radical activity in blacks with chronic pancreatitis at Johannesburg, South Africa. *Clinica Chimica Acta* . **230(2):** 189-199.

Hadler, H.I. (1989): Comment: mitochondrial genes and cancer. *FEBS Letters.* **256(1 and 2):** 230-231.

Hadler, H.I., Daniel, B.G. and Pratt, R.D. (1971): The induction of ATP energized mitochondrial volume changes by carcinogenic N-hydroxy-N-acetyl-aminofluorenes when combined with showdomycin. A unitary hypothesis for carcinogenesis. *Journal of Antibiotics.* **24(7)**: 405-417.

Halliwell, B., and Gutteridge, J.M.C. (1985): The importance of free radicals and catalytic metal ions in human disease. *Mol. Aspects Med.* **8:** 89-193.

Hannon, M.R., Greichus, Y.A., Applegate, R.L., and Fox, A.C. (1970): Ecological distribution of pesticides in Lake Poinsett, South Dakota. Trans. *Amer. fish Soc.* **99:** 496-499.

Harman, D. (1968): Free radical theory of aging: Effect of free radical reaction inhibitors on the mortality rate of male LAF mice. *J. Gerontol.* **23:** 476-482.

Harvey, P.R.C., Ilson, R.G. and Strasberg, S.M. (1989) The simultaneous determination of oxidized and reduced glutathiones in liver tissue by ion pairing reverse phase high performance liquid chromatography with a coulometric electrochemical detector. *Clinica Chimica Acta.* **180**: 203-212

Hassan, M.Q., Numan, I.T., Al-Nasiri, N., and Stohs, S.J. (1991): Endrin-induced histopathological changes and lipid peroxidation in livers and kidneys of rats, mice, guinea pigs and hamsters. *Toxicologic Pathology.* **19(2):** 108-114.

Hayes, W.J. and Curley, A. (1968): Storage and excretion of dieldrin and related compounds. Effect of occupational exposure. *Arch. Envirom. Hlth.* **16:** 155-162.

Hendrich, S., Krueger, K., H. W. Chen and Cook, L (1991): Phenobarbital increases rat hepatic prostaglandin F2alpha, Glutathione S-Transferase

Activity and Oxidative Stress. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **42**: 45-50.

Hewett, J.A. and Roth, R.A. (1988): Dieldrin activates rat neutrophils in vitro. *Toxicology and Applied Pharmacology* **96**: 269-278.

Hinrichsen, L.I., Floyd, R.A., and Sudilovsky, O. (1990): Is 8-hydroxydeoxyguanosine a mediator of carcinogenesis by a choline-devoid diet in the rat liver? *Carcinogenesis.* **11(10):** 1879-1881.

Hochstein, P. and Atallah, A.S. (1988): The nature of oxidants and antioxidant systems in the inhibition of mutation and cancer. *Mutation Res.* **202:** 363-375.

Horio, F., Kimura, M., and Yoshida. A. (1983): Effect of several xenobiotics on the activities of enzymes affecting ascorbic acid synthesis in rats. *Journal of Nutritional Science and Vitaminology*. **29(3):** 233-247.

Hruszkewycz, A.M. (1992): Lipid peroxidation and mtDNA degeneration. a hypothesis. *Mutation Research*. **275:** 243-248.

Huang, X., Zhuang, Z., Frenkel, K., Klein, C.B. and Costa, M. (1994): The role of nickel and nickel-mediated reactive oxygen species in the mechanism of nickel carcinogenesis. *Environmental Health Perspectives*. **102(3):** 281-284.

Hutson, D.H. (1975): Comparative metabolism of dieldrin in the rat (CFE) and in two strains of Mouse (CF1 and LACG). *Food and Cosmetic Toxicology*. **14**: 577-591.

IARC monographs, 5, 125-156 (1974)

Ikegaki, N., Katsumata, M., Minna, J., and Tsujimoto, Y. (1994): Epression of bcl-2 in small cell lung carcinoma cells. *Cancer Research*. **54**: 6-8.

Intracellular sorting and the maintenance of cellular compartments. In: Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. Eds. Molecular Biology of the Cell, second edition. (1989) New York. Garland Publishing, Inc. pp 431-432.

Izushi, F. and Ogata, M. (1990): Hepatic and muscle injuries in mice treated with heptachlor. *Toxicology Letters*. **54:** 47-54.

Jakoby, W.B. (1978): The glutathione S-transferases: A group of multifunctional detoxification proteins. *Adv. Enzymol.* **46:** 383-414.

Jones, D. Thor, M., Smith, M., Jewell, S., and Orrenius, S. (1983): Inhibition of ATP dependent microsomal Ca2+ sequestion during oxidative stress and its prevention by glutathione. *Journal of Biological Chemistry.* **258**: 6390-6393.

Junqueira, B.C.V., Simizu, K., Videla, L.A. and Barros, S.B. (1986): Dose dependent Study of the effects of acute lindane administration on rat liver superoxide anion production, antioxidant enzyme activitities and lipid peroxidation. *Toxicology.* **41**:198-204.

Junqueira,V.B., Simizu,K., Pimental, R., Azzalis, L.A., Barros, S.B., Koch, O. and Videla, L.A. (1991): Effect of phenobarbital and 3-methylcholanthrene on the early oxidative stress component induced by lindane in rat liver. *Xenobiotica.* **21(8):** 1553-1065.

Kaplowitz, N., Aw, T.Y. & Ookhtens, M. (1985): The regulation of hepatic glutathione. *Ann. Rev. Pharmacol. Toxicol.* **25**:715-744

Kappius, H. and Diplock, A.T. (1992): Tolerance and safety of Vitamin E: A toxicological position report. *Free Radical Biology and Medicine.* **13**: 55-74.

Kasai, H., Crain, P.F., Kuchine. Y., Nishimura, S., Ootsuyama, A., and Tanooka, H. (1986): Formation of 8-hydroxyguanine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair. *Carcinogenesis.* **7(11):** 1849-1851.

Kass, G.E.N., Duddy, S.K. and Orrenius, S. (1989): Alterations of hepatocyte protein kinase C by redox-cycling quinones. *Biochem, J.* **260:** 499-507.

Katsumoto, F., Miyazaki, K., and Nakayama, F. (1989): Stimulation of DNA synthesis in hepatocytes by Kupffer cells after partial hepatectomy. *Hepatology.* **9(3):** 405-410.

Kefalas, V. and Stacey, N.H. (1989): Potentiation of carbon tetrachloride-induced lipid peroxidation by trichloroethylene is isolated rat hepatocytes: no role in enhanced toxicity. *Toxicology and Applied Pharmacology.* **101**: 158-169.

Kehrer, J.P. (1993): Free radicals as mediators of tissue injury and disease. *Critical Reviews in Toxicology.* **23(1)**: 21-48.

Kensler, T.W. and Trush, M.A. (1984): Role of oxygen radicals in tumor promotion. *Environmental Muitagenesis.* **6**: 593-616.

Klaunig, J.E. (1991): Alterations in intercellular communication during the stage of promotion. Society for Experimental Biology and Medicine. pp688-692.

Klaunig, J.E., Goldblatt, P.J., Hinton, D.E., Lipsky, M.M., Chacko, J. and Trump, B.F. (1981): Mouse liver cell culture. I. Hepotocyte isolation. *In Vitro.* **17**(10): 913-925.

Klaunig, J.E., Ruch, R.J., Hampton, J.A., Weghorst, C.M., and Hartnett, J.A. (1990): Gap-junctional intercellular communication and murine hepatic carcinogenesis. mouse liver carcinogenesis: mechanisms and species Comparisons. Alan R. Liss, Inc., NY pp277-291.

Klaunig,J.E. and Baker,T.K. (1994) Morphological: evaluation of gap junctional intercellular communication. In C.A. Tyson and J.M. Frazier (eds) Methods In Toxicology: In Vitro Toxicity Indicators, Academic Press, San Diego, CA vol. II, pp 72-80.

Klein-Szanto, A.J.P and Slaga,T.J (1982): Effects of peroxides on rodent skin epidermal hyperplasia and tumor promotion. *Journal of Investigative Dermatology.* **79:** 30-34.

Knekt, P., Aromaa, a., Maatela, J., Aaran, R-K., Nikkari, t., Hakama, M., Hakulinen, T., Peto, R., and Teppo, L. (1991): Vitamin E and cancer prevention. *Am J. Clin. Nutr.* **53:** 283S-286S.

Kohli, K.K., Maggon, K.K. and Venkitasubramanian, T.A. (1977): Induction of Mixed Function Oxidases on Oral Administration of Dieldrin. *Chem. Biol. Interactions.* **17**:249-255.

Kretsinger, R.H. (1980): Structure and evolution of calcium-modulated proteins. *CRC Critical Reviews in Biochemistry.* **8(2):** 119-174.

Ku, H-H.K., Brunk,U.T., Sohal, R.S. (1993): Relationship between mitochondrial superoxide and hydrogen peroxide production and longevity of mammalian species. *Free Radical Biology and Medicine. **15**: 621-627.*

Lake, B.G., Evans, J.G., Walters, D.G. and Price, R.J. (1991): Comparison of the hepatic effect of nafenopin, a peroxisome proliferator, in rats fed adequate or vitamin E-- and selenium-- deficient diets. *Human Exp. Toxicol.* **10:** 87-88.

Laskin, D.L. and Pilaro, A. (1986): Potential role of activated macrophages in acetaminophen hepatotoxicity. I. Isolation and characterization of activated macrophages from rat liver. *Toxicol. Appl. Pharmacol.* **86**: 204-215.

Laskin, D.L., Robertson, F.M., Pilaro, A.M. and Laskin, J.D. (1988): Activation of liver macrophages following phenobarbital treatment of rats. *Hepatology.* **8**: 1051-1055.

Lawrence, R.A. and Burk, R.F. (1976): Glutathione peroxidase activity in selenium deficient rat liver. *Biochem. Biophys. Res. Comm.* **71(4)**: 952-958.

Lecomte, E., Herbeth, B., Pirollet, P., Chancerelle, Y., Arnaud, J., Musse, N., Paille, F., Siest, G., and Artur, Y. (1994): Effect of alcohol consumption on blood antioxidant nutrients and oxidative stress indicators. *American Journal of Clinical Nutrition.* **60(2)**: 255-261.

Ledda-Columbano, G.M., Columbano, A., Cannas, A., Simbula, G., Okita, K., Kayano, K., Kubo, Y., Katyal, S.L. and Shinozuka, H. (1994): Dexamethasone inhibits induction of liver tumor necrosis factor-alpha mRNA and liver growth induced by lead nitrate and ethylene dibromide. *American Journal of Pathology.* **145(4)**: 951-8.

Lickl, E., Alth, G., Ebermann, R., Beck, R.H., Tuma, K., (1989): Serum urate level and cancer. *Medical Hypotheses.* **28(3)**: 193-195.

Loewenstein, W.R. (1979): Junctional intercellular communication and the control of growth. *Biochim. Biophys. Acta.* **560**: 1- 65.

Loury, D.J., Goldworthy, T.L, and Butterworth, B.E. (1987): The value of measuring cell replication as a predictive index of tissue-specific tumorigenic potential In. B.E. Butterworth and T.J. Slaga (Eds.) Nongenotoxic Mechanisms in Carcinogenesis. [Banbury Report 25] Cold Spring Harbor Laboratory, Cold Spring Harbor, pp. 119-136.

Lubet, R.A., Nims, R.W., Ward, J.M., Rice, J.M., and Diwan, B.A. (1989): Induction of cytochrome P-450 and its relationship to liver tumor promotion. *Journal of the American College of Toxicology.* **8(2)**: 259-268.

Lubet, R.A., Syi, J-L, Nelson, J.O., and Nims, R.W. (1990): Induction of hepatic cytochrome P-450 mediated alkoxyresorufin o-dealkylase activities in different species by prototype P-450 inducers. *Chem. Biol. Interactions.* **75**: 325-339.

Lucier, G.W., Tritscher,A., Goldsworthy,T., Foley,J., Clark,G., Goldstein,J., and Maronpot,R. (1991): Ovarian hormones enhance TCDD-mediated increases in cell proliferation and preneoplastic foci in a two stage model for rat heptocarcinogensis. *Cancer Res.* **51:** 1391-1397.

Magee, P.N. (1974): Activation and inactivation of chemical carcinogens and mutagens n the mammal. *Essays in Biochemistry*. **10**:105-136.

Mahoney, C.W. and Azzi, A. (1988): Vitamin E inhibits protein kinase C activity. *Biochemical and Biophysical Research Commun ication*. **154(2):** 694-697.

Marcus, R. and Coulston, A.M. (1993): The Vitamins. In: Gilman, A.G., Rall, T.W., Nies, A.S. and Taylor, P. Ed. The Pharmacological Basis of Therapeutics. New York: McGraw-Hill, Inc. 1523-1571.

Marklund, S. and Marklund, G. (1974): Involvement of the superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for superoxide dismutase. *Journal of Biochemistry* . **47**: 469-474.

Marmur, J. (1961): A procedure for the isolation of deoxyribonucleic acid from microorganisms, *J. Mol. Biol.* **3**: 208-216

Marsman, D.S., Cattley, R.C., Conway, J.G. and Popp, J.A. (1988): Relationship of hepatic peroxisome proliferation and replicative DNA synthesis to the hepatocarcinogenicity of the peroxisome proliferators di(2-ethylhexyl)phthalate (DEHP) and [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (Wy 14,643) in rats. *Cancer Res.* **48:** 6739-6744.

Masaki, N. Thomas, A.P., Hoekj, J.B. and Farber, J.L. (1989): Intracellular acidosis protects cultured hepatocytes from the toxic consequences of a loss of mitochondrial energization. *Archives of Biochemistry and Biophysics.* **272**:152-161.

Masters, C.J. and Crane, D.I. (1995): On the role of the peroxisome in ontogeny, aging and degenerative disease. *Mechanisms of Aging and Development*. **80:** 69-83.

Mathews, C.K. and van Holde, K.E. (1990): BioChemistry. The Benjamin/Cummings Publishing Company. Redwood City, CA.

McCord, J.M. (1993): Human disease, free radicals, and the oxidant/antioxidant balance. *Clin. Biochem.* **26:** 351-357.

McCord, J.M. and Fridovich, I. (1969): The utility of superoxide dismutase in studying free radical reactions. I. Radicals generated by the interaction of sulfite, dimethyl sulfoxide, and oxygen. *Journal of Biological Chemistry.* **244(22)**: 6056-63.

Melnick, R.L. (1992): Does chemically induced hepatocyte proliferation predict liver carcinogenesis? *The FASEB Journal.* **6:** 2698-2706.

Mitchel, R.E.J. and McCann, R. (1993): Vitamin E is a complete tumor promoter in mouse skin. *Carcinogenesis.* **14(4):** 659-662.

Monroe, D.H. & Eaton, D. (1987): Comparative effects of butylated hydroxyanisole on hepatic in vivo DNA binding and in vitro biotransformation of aflatoxin B1 in the rat and mouse. *Toxicol. & Appl. Pharmacol.* **90**:401-409

Moody, D.E., Montigomery, K.A., Ashour, M.B. and Hammock,B.D. (1991): Effects of environmentally encountered epoxides on mouse liver epoxide-metabolizing enzymes. *Biochemical Pharmacology* **41(11)**: 1625-37.

Moser, G.J. and Smart R.C. (1981): Hepatic tumor promoting chlorinated hydrocarbons stimulate protein kinase C activity. *Carcinogenesis* **10(5)**:851-856.

Mukai,F.H. and Goldstein, B.D. (1976): Mutagenicity of malonaldehyde, a decomposition product of peroxidized polyunsaturated fatty acids. *Science.* **19(4229):** 868-869

Nathan, C.F. and Tsunawake, S. (1986): Secretion of toxic oxygen products by macrophages: regulatory cytokines and their effects on the oxidase. *Ciba foundation Symposium.* **118:** 211-230.

Neal, G.E., Nielsch, U., Judah, D.J. & Hulbert, P.B. (1987): Conjugation of model substrates or microsomally-activated Aflatoxin B1 with reduced glutathione, catalyzed by cytosolic glutathione-s-transferases in livers of rats, mice and guinea pigs. *Biochemical Pharmacology.* **36**:4269-4276

Nerurkar,P.V., Park,S.S.,Thomas,P.E., Nims, R.W., and Lubet, R.A. (1993): Methoxyresorufin and benzyloxyresorufin: substrates preferentially metabolized by cytochromes P450 1A2 and 2B, respectively, in the rat and mouse. *Biochemical Pharmacology.* **46(5):** 933-943.

Neubert,D., Hopfenmuller, W., and Fuchs, g. (1981): Manfestation of carcinogenesis as a stochastic process on the basis of an altered mitochondrial genome. *Arch. Toxicol.* **48**: 89-125.

Neubert,D., Wojtszak, A.B., and Lehninger, A.L. (1962): Purification and enzymatic identity of mitochondrial contraction-factors I and II. *Proc. Natl. Acad. Sci. U.S.A.* **48:** 1651-1658.

Nicotera, T.M.(1991): Molecular and biochemical aspects of Bloom's syndrome. *Cancer Genetics & Cytogenetics.* **53(1):** 1-13.

NiKi, E. (1991): Vitamin C as an antioxidant (review). *World Review of Nutrition & Dietetics.* **64**:1-30

Nohl, H., De Silva, D., Summer, K-H., (1989): 2,3,7,8-tetrachlorodibenzo-p-dioxin induces oxygen activation associated with cell respiration. *Free Radical Biol. Med.* **6:** 369-374.

NTP Technical report on the toxicology and carcinogenesis studies of tetrachloroethylene (perchloroethylene) in F344/N rats and B6C3F1 mice (inhalation studies) DHSS-NIH report 85-2657 Washington D.C. Department of Health and Human Services (1988).

NTP Toxicology and Carcinogenesis studies of dichloromethane (methylene chloride) in F344/N rats and B_6C_3F1 mice (inhalation studies) National Toxicology Program Report NTP TR 306 (1986).

Nutter, L.M., Wu,Y-Y., Ngo, E.O., Sierra, E.E., Gutierrex, P.L. and Abul-Hajj, Y.J. (1994): An o-Quinone form of estrogen produces free radicals in human breast cancer cells: correlation with DNA Damage. *Chem. Res. Toxicol.* **7(1):** 23-28.

Nyandieka, H.S., Wakhis, j., and Kilonzo, M.M. (1990): Association of reduction of AFB1-induced liver tumours by antioxidants with increased activity of microsomal enzymes. *Indian Journal of Medical Research.* ***92:*** *332-336.*

Oberholser, K.M. Wagner, S.R. and Greene F.E. (1977): Factors affecting dieldrin metabolism by rat liver Microsomes. *Drug Metabolism and Disposition* **5(3)**:302-309.

Ono, J. Mimake, T. and Yabuuchi, H. (1986): Effects of phenobarbital on lipid peroxidation in vitamin E deficient rats. *Pediatric Pharmacology* **5**:223-227.

Pacifici, R.E. and Davies, K.J.A. (1991): Protein, lipid and DNA repair systems in oxidative stress: the free-radical theory of aging revisited. *Gerontology*. **37:** 166-180.

Palmer, R.M.J., Ashton,D.S. and Moncada, S. (1988): Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*. **333:** 664-666.

Parke, D.V., and Ioannides, C., (1990): Role of Cytochrome P-450 in mouse liver tumor production. mouse liver carcinogenesis: Mechanisms and species comparisons, p215-230 Alan R. Liss, Inc

Pathak, M.A., and Stratton, K. (1968): Free radicals in human skin before and after exposure to light. *Arch. Biochem*. **123:** 468-476.

Pauling, L. (1991): Effect of ascorbic acid on incidence of spontaneous mammary tumors and UV-light induced skin tumors in mice. *Am. J. Clin. Nutr*. **54:** 1252S-1255S.

Pearl, A., Hale, A., and Whitehead. T. (1993): Serum urate as a free radical sacavenger in diabetics. *Journal of Medical Systems*. **17(3-4):** 233-237.

Perchellet, J.P., Owen, M.D., Posey, T.D., Orten, D.K., Schneider, B.A. (1988): Inhibitory effects of glutathione level-raising agents and D-alpha-tocopherol on ornithine decarboxylase induction and mouse skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate. *Carcinogenesis*. **6(4):** 567-573.

Pronzato, M.A., Domonicotti, C., Rosso, E., Bellocchio, A., Patrone, M., Marinari, U.M., Melloni, E., and Poli, G. (1993): Modulation of rat liver protein kinase C during "in vivo" CCl4-induced oxidative stress. *Biochemical and Biophysical Research Communication*. **194(2)**:635-60.

Quiroga,, G.B., Lopez-Torres, M., Perez-Campo,R. and Rojas,C. (1991): Simultaneous determination of two antioxidants, uric and ascorbic acid, in animal tissue by high-performance liquid chromatography. *Analytical Biochemistry*. **199:** 81-95.

Radi, R., Beckman, J.S., Bush, K.M., and Freeman, B.A. (1991): Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic otential of superoxide and nitric oxide. *Archives of Biochemistry and Biophysics* **288(2):** 481-487.

Radler-Pohl, A., Gebel, S., Sachsenmaier, C., Kinig, H., Kramer, M., Oehler, T., Streile, M., Ponta, H., Rapp, U., Rahmsdorf, H. J., Cato, A.C.B., Angel, P.,

and Herrlich, P. (1993): The activation and activity control of AP-1 (Fos/Jun). *Annals New York Academy of Sciences* **684**:127-148.

Rao, M.S. and Reddy, J.K. (1991): An overview of peroxisome proliferator-induced hepatocarcinogenesis. *Environmental Health Perspectives.* **93:** 205-209.

Ray,J.S., Harbison, M.L., McClain, R.M., and Goodman, J.I. (1994): Alterations in the methylation status and expression of the raf oncogene in phenobarbital-induced and spontaneous B6C3F1 mouse liver tumors. *Molecular Carcinogenesis.* **9(3):** 155-166.

Reddy, J.K. and Rao, M.S. (1977): Malignant tumors in rats fed nafenopin, a hepatic peroxisome proliferator. *J. Natl. Cancer Inst.* **59:** 1645-1650.

Reddy, J.K. and Qureshi, S.A. (1979): Tumorigenicity of the hypolipidaemic peroxisome proliferator ethyl-a-chlorophenoxy-isobutyrate (clofibrate) in rats. *Br. J. Cancer.* **40**: 476-482.

Reed, D.J. (1990): Review of the current status of calcium and thiols in cellular injury. *Chem. Res Toxicol.* **3:** 495-502.

Reiners, J.J., Thai, G., Rupp, T., and Cantu, A.R. (1991): Assessment of the antioxidant/prooxidant status of murine skin following topical treatment with 12-O-tetradecanoylphorbol-13-acetate and throughout the ontogeny of skin cancer. Part I: quantitation of superoxide dismutase, catalase, glutathione peroxidase and xanthine oxidase. *Carcinogenesis.* **12(12):** 2337-2343.

Richie, J.P., (1992): The Role of Glutathione in aging and Cancer. *Experimental Gerontology.* **27**:615-626.

Richter, C. (1992): Reactive Oxygen and DNA damage in mitochondria. *Mutation Research.* **275**: 249-255.

Ruch, R.J. and Klaunig, J.E. (1988): Inhibition of Mouse Hepatocyte Intercellular Communication by Paraquat-Generated Oxygen Free Radicals. *Toxicology and Appplied Pharmacology.* **94:** 427-436.

Ruch, R. J., Bandyopadhyay, S., Somani, P. and Klaunig, J.E. (1991): Evaluation of amiodarone free radical toxicity in rat hepatocytes. *Toxicology Letters.* **56**:117-126.

Sadrzadeh, S.M. and Nanjj, A.A. (1994): Detection of lipid peroxidation after acute ethanol administration is dependent on time of sampling. *International Journal for Vitamin and Nutrition Research*. **64(2):** 178-158.

Sand, P.F., Wiersma, G.B., and Landry, J.L. (1972): Pesticide residues in sweet potatoes and soil -1969. *Pest. Monit. J.* **5:** 342-344.

Sandy, M.S., Di monte, D., and Smith, M.T. (1990): Relationships between intracellular vitamin E, lipid peroxidation, and chemical toxicity in hepatocytes. *Toxicology and Applied Pharmacology*. **93(2):** 288-297.

Sandy, M.S., Moldeus, P., Ross, D., and Smith, M.T. (1987): Cytotoxicity of the redox cycling compound diquat in isolated hepatocytes: involvement of hydrogen peroxide and transition metals. *Archives of Biochemistry and Biophysics*. **259(1):** 29-37.

Sauberlich, H.E. (1994): Pharmacology of Vitamin C. *Annual Review of Nutrition*. **14:** 371-91.

Schreck, R., Albermann, K., and Baeuerle, P.A. (1992): Nuclear Factor kB: An oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free Radical Research Communication*. **17:** 221-237.

Schulte-Hermann, R. (1974): Induction of liver growth by xenobiotic compounds and other stimuli. *CRC Critical Reviews in Toxicology*. 97-158.

Schulte-Hermann, R.S. (1987): Tumor promotion in the liver. *Archiv. Toxicol*. **57:** 147-158.

Schumann, A.M., Quast, J.F., Watanabe, P.G. (1980): The pharmacokinetics and macromolecular interactions of perchloroethylene in mice and rats as related to oncogenicity. *Toxicol. Appl. Pharmacol*. **55:** 207-219.

Schwartz, J.L., Antoniades, D.Z., and Zhao, S. (1993): Molecular and biochemical reprogramming of oncogenesis through the activity of prooxidants and antioxidants. *Annals of the New York Academy of Science.* **686:** 262-278.

Shay, J.W. and Werbin, H. (1992): New evidence for the insertion of mitochondrial DNA into the human genome: significance for cancer and aging. *Mutation research*. **275**: 227-235.

Shigenaga, M.K. and Ames. B.N. (1991): Assays for 8-hydroxy-2'-deeoxyguanosine: a biomarker of in vivo oxidative DNA damage. *Free Radical Biology and Medicine.* **10:** 211-216.

Shima, A. and Sugahara, T. (1976): Age-dependent ploidy class changes in mouse hepatocyte nuclei as revealed by Feulgen-DNA cytofluorometry. *Exp. Geront.* **11:** 193-203.

Shiratori, Y., Takikawa, H., Kawase, T., Sugimoto, T. (1986): Superoxide anion generating capacity and lysosomal enzyme activities of kupffer cells in galactosamine-induced hepatitis. *Gastroenterology Jpn.* **21:** 135-144.

Sies, H (1991): Oxidative Stress: Introduction. In Sies, H. (ed): Oxidative Stress: Oxidants and Antioxidants. San Diego, Academic Press pp 15-22.

Sies, H. and Murphy, M.E. (1991): Role of tocopherols in the protection of biological systems against oxidative damage. *J. Photochem. Photobiol. B: Biol.* **8**: 211-224.

Simic, M.G. (1994): DNA markers of oxidative processes in vivo: relevance to carcinogenesis and anticarcinogenesis. *Cancer Research (suppl.)* **54**: 1918s-1923s.

Sipes, I.G. and Gandolfi, A.J. !990): Biotransformation of Toxicants. In: Amdur, M. O., Doull, J., and Klaassen, C.D. Toxicology 4th edition. (1991) New York. Pergamon Press.

Slaga,T.J., Klein-Szanto, A.J.P., Triplett, L.L. Yotti,L.P., and Trosko,J.E. (1981): Skin tumor promoting acitvity of benzoyl peroxide: a widely used free radical generating compound. *Science.* **213:** 1023-1025.

Smith, R.C., and Lawing, L. (1983): Antioxidant activity of uric acid and 3-N-ribosyluric acid with unsaturated fatty acids and erythrocyte membranes. *Arch. Biochem. Biophys.* **223:** 166-172.

Smith-Oliver, T. and Butterworth, B.E. (1987): Correlation of the carcinogenic potential of di(2-ethylhexyl)phthalate (DEHP) with induced hyperplasia rather than with genotoxic activity. *Mutat. Res.* **188:** 21-28.

Sohal, R.S. and Dubey, A. (1994): Mitochondrial oxidative damage, hydrogen peroxide release and aging. *Free Radical Biology and Medicine.* **16(5):** 621-626.

Sohal, R.S., Svensson, I. and Brunk, U.T. (1990): Hydrogen peroxide production by liver mitochondria in different species. *Mechanisms of Aging and Development*. **53**: 209-215.

Solt, D. and Farber, E. (1976): New principle for the analysis of chemical carcinogenesis. *Nature*. **263**: 701-703.

Sorta, G., Tartaglia, L.A., Ames, B.N. (1990): Transcriptional regulator of oxidative stress inducible genes: direct activation by oxidation. *Science*. (Washington DC) **248**: 189-194.

Stevenson, D.E., Kehrer, J.P., Kolaja, K.L., Walborg, E.F. and Klaunig, J.E. (1995): Effect of dietary antioxidants on dieldrin-induced hepatotoxicity in mice. *Toxicology Letters*. **75(1-3)**: 177-183.

Stohs,, S.J., Shara, M.A., Alsharif, N.Z., Wahba, Z.Z. and Al-Bayati, Z.A.F. (1990): 2,3,7,8-Tetrachlorodibenzo-p-dioxin-Induced Oxidative Stress in Female Rats. *Toxicology and Applied Pharmacology*. **106**: 126-135.

Stohs, S.J., Alsharif, N.Z., Shara, M.A., Al-Bayati, Z.A.F. and Wahba, Z.Z. (1990): Evidence for the induction of an oxidative stress in rat hepatic mitochondria by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Biological Reactive Intermediates IV, Edited by C.M. Witmer et al. Plenum Press, New York.

Strasser, A., Harris, A.W., Cory, M. S. (1991): Bcl-2 transgene inhibits T cell death and perturbs thymic self censorship. *Cell*. **67**: 889-899.

Sun, Y. (1990): free radicals, antioxidant wnzymes, and carcinogenesis. *Free Radical Biology & Medicine*. **8:** 583-599.

Suzuki, Y.J. and Packer, L. (1993) inhibition of NF-kB activation by vitamin e derivatives. *Biochemical and Biophysical Research Communications.* **193(1):** 277-283.

Swann, J.D., Smith, M.W., Phelps, P.C. Maki, A. Berezesky, I.K. and Talalay, P.(1988): Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis. *Prot. Natl. Acad. Sci. USA.* **85**: 8261-8265.

Swann, J.D., Smith, M.W., Phelps, P.C. Maki, A. Berezesky, I.K. and Trump, B.F. (1991): Oxidative injury induces influx-dependent changes in intracellular calcium homeostasis. *Toxicologic Pathology*. **19(2):** 128-137.

Talalay, P., De Long, M.J., and Prochaska, H.J. (1988): Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis. *Proc. Natl. Acad. Sci., USA.* **85:** 8261-8265.

Tamura, H., Lida, T., Watanabe, T. and Suga, T. (1990): Long term effects of hypolipidaemic peroxisome proliferator administration on hepatic hydrogen peroxide metabolism in rats. *Carcinogenesis.* **11:** 445-450.

Tennekes, H.A., Edler, L. and Kunz, H.W. (1982): Dose response analysis of the enhancement of liver tumour formation in CF-1 mice by dieldrin. *Carcinogenesis.* **3(8):** 941-945.

Thorpe, E. and Walker, A.I. (1973): The Toxicology of dieldrin (HEOD). II comparative long-term oral toxicity studies in mice with dieldrin, DDT, phenobarbitone, B-BHC and gamma-BHC. *Food and Cosmetic Toxicology*, **11(3):** 433-442.

Tilbury, L., Butterworth, B.E., Moss,O., and Goldsworthy, T.L. (1993): Hepatocyte cell proliferation in mice after inhalation exposure to unleaded gasoline vapor. *J. Toxicol. Environ. Health.* **38**: 293-307.

Tilly, J.L. and Tilly, K.I. (1995): Inhibitors of oxidative stress mimic the ability of follicle-stimulating hormones to suppress apoptosis in cultured rat ovarian follicles. *Endocrinology.* **136(1):** 242-252.

Tolbert, N.E. and Essner, E. (1981): Microbodies: peroxisomes and glyoxysomes. *J. Cell. Biol.* **91:** 271s-283s.

Tomaszewski, K.E., Heindel, S.W., Jenkins, W.L. and Melnick, R.L. (1990): Induction of peroxisomal acyl CoA oxidase activity and lipid peroxidation in primary rat hepatocyte cultures. *Toxicology.* **65:** 49-60.

Tomatis, L., Partansky, C., and Montasano, R. (1973): The predictive value of mouse liver tumour induction in carcinogenicity testing--a literature survey. *International Journal of Cancer.* **12(1):** 1-20.

Trickler, D. and Shklar, G. (1987): Prevention by vitamin E of experimental oral carcinogenesis. *Journal of the National Cancer Institute.* **78(1):** 165-169.

Troll, W. and Wiesner, R (1985): The role of oxygen radicals as a possible mechanism of tumor promotion. *Ann. Rev. Pharmacol. Toxicol.* **25**: 509-528.

Trosko, J.E. and Chang C.C. (1984): Adaptive and nonadaptive consequences of chemical inhibition of intercellular communication. *Pharmacological Reviews*. **36(2 Suppl):** 137S-144S.

Trush, M.A. and Kensler, T.W. (1991): An overview of the relationship between oxidative stress and chemical carcinogenesis. *Free Radical Biology and Medicine*. **10**:201-209

Tse, W.Y., Maxwell, S.R., Thomason, H., Blann, A., Thorpe, G.H., Waite, M., and Holder, R. (1994): Antioxidant status in controlled and uncontrolled hypertension and its relationship to endothelial damage. *Journal of Human Hypertension*. **8(11):** 843-849.

Uchiyama, M. and Mihara, M. (1978): Determination of Malonaldehyde Precursor in Tissues by Thiobarbituric Acid Test. *Analytical Biochemistry*. **86**: 271-278.

US Environmental Protection Agency (1973) Environmental News, August 3, Washington DC, US Government Printing Office

US Tariff Commission (1951): Synthetic Organic Chemicals, United States Production and Sales, 1950 Second Series, Report No. 173, Washington DC, US Government Printing Office, p 127.

van Ravenzwaay, B., and Kunz, W. (1988): Quantitative aspects of accelerated nuclear polyploidization and tumour formation in dieldrin treated CF-1 mouse liver. *Br. J. Cancer*. **58**: 52-56.

van Ravenzwaay, B., Toussaint, H.J.M. and Schmitt, R.L. (1988): Dieldrin-induced changes in isoenzyme composition in the livers of CF-1 mice. *Int. J. Cancer*. **41**: 305-308.

Vericel, E., Narce, M., Ulmann, L., Poisson, J-P., and Lagarde, M. (1994): Age-related changes in antioxidant defense mechanisms and peroxidation in isolated hepatocytes from spontaneously hypertensive and normotensive rats. *Molecular and Cellular Biochemistry*. **132:** 25-29.

Vina, J., Sastre, J., Anton, V., Bruseghini, L., Esteras, A. and Asensi, M. (1992): Effect of aging on glutathione metabolism. Protection by antioxidants. *Free Radicals and Aging*. **62:** 136-144.

Vo.,T.K.-O., Druez,C., Delzenne,N. Taper, H.S., and Roberfroid,M. (1988): Analysis of antioxidant defense systems during rat hepatocarcinogenesis *Carcinogenesis*. **9(11)**:2009-2013.

Vos, R.M.E. and Bladeren, P.J. (1990): Glutathione S-transferases in relation to their role in biotransformation of xenobiotics. *Chem. Biol. Interactions.* **75:** 241-265.

Wada, N., Marsman, D.S. and Popp, J.A. (1992): Dose related effects of hepatocarcinogen Wy 14,623 on peroxisomes and cell replication. *Fundam. Appl. Toxicol.* **18:** 149-154.

Walker,A.I.T., Thorpe, E. and Stevenson, D.E. (1972): The toxicology of Dieldrin (HEOD). I. Long-term oral toxicity studies in Mice. *Food and Cosmetic Toxicology* **11**: 415-432.

Walker,A.I.T., Stevenson, D.E., Robinson, J., Thorpe, E. and Roberts, M. (1969): The toxicology and pharmacodynamics of dieldrin (HEOD): two year oral exposure of rats and dogs.. *Toxicology and Applied Pharmacology* **15**: 345-373.

Washko, P.W., Hartzell, W.O. and Levine, M. (1989): Ascorbic acid analysis using high-performance liquid chromatography with coulometric electrochemical detection. *Anal. Biochemistry*. **181**:276-282

Wefer, A., and Sies, H. (1988): The protection by ascorbate and glutathione against microsomal lipid peroxidation is dependent on Vitamin E. *Eur. J. Biochem*. **174**: 353-357.

Weisigen, and Fridovich (1973): Mitochondrial superoxide dismutase. Site of synthesis and intramitochondrial localization. *Journal of Biological Chemistry*. **248(13)**: 4793-6.

Weiss, S.J. and LoBuglio, A.F. (1982): Phagocyte-generated oxygen metabolites and cellular injury. *Lab Invest*. **47:** 5-17.

Williams, G.M. (1981): Liver Carcinogenesis: the Role for some Chemicals of an Epigenetic Mechanism of Liver-Tumour Promotion involving Modification of the Cell Membrane. *Fd. Cosmet. Toxicol.* **19:** 577-581.

Williams, G.M. and Numoto, S. (1984): Promotion of mouse liver neoplasms by the organochlorine pesticides chlordane and heptachlor in comparison to dichlorodiphenyltrichloroethane. *Carcinogenesis*. **5(12)**: 1689-1696.

Wilson, D.M., Goldsworthy, T.L., Popp, J.A., and Butterworth, B.E. (1992): Evaluation of genotoxicity, pathological lesions, and cell proliferation in

livers of rats and mice treated with furan. *Environ. Mol. Mutagen.* **19:** 209-222.

Wong, D.Y., Hsiao, Y.L., Poon, C.K. , Kwan, P.C., Chao, S.Y., Chow, S.T. and Yang, C.S. (1994): Glutathione concentration in oral cancer tissues. *Cancer Letters.* **81(2):** 111-116.

Wright, A.S., Potter,D., Wooder, M.F., Donninger, C. and Greenland R.D. (1972): The effects of dieldrin on the subcellular structure and function of Mammalian liver Cells. *Food and Cosmetic Toxicology* **10**:311-332 Pergamon Oress.

Wunderlich, V., Tetzlaff,I., and Graffi, A. (1971): Studies on nitrosodimethylamine: preferential methylation of mitochondrial DNA in rats and hamsters. *Chem. Biol. Interactions.* **4**: 81-89.

Wyllie, A.H. (1992): Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: an overview. *Cancer and Metastasis Reviews.* **11**: 95-10

Yalcin, S., Jensson, H., and Mannervik, B. (1983): A set of inhibitors for discrimination between the basic isozymes of glutathione transferase in rat liver. *Biochemical and Biophysical Research Communications.* **114(2):** 829-834.

Yamasaki, H. (1988): Tumor promotion: from the viewpoint of cell society. In: O.H. Iversen (Ed.), Theories of Carcinogenesis. Hemisphere Publishing Corp. Washington D.C. pp. 143-157.

Zhan, H., Sun C-P., Liu, C-G. & Zhou, J-H. (1992) Age-related change of free radical generation in liver and sex glands of rats. *Mechanisms of Aging and Development.* **62(2)**: 111-116.

NAME: Stephen Bachowski, MAJOR, USAF

1995: Research Toxicologist
Tri-Service Toxicology Center
Wright-Patterson Air Force Base, Ohio

Work Experience

1978-1981 Research and Development Organic Chemist for Eastman Kodak Company

Responsible for developing chemical processes to produce organic macro molecules used in photographic films. Beside the obvious knowledge required as an organic chemist, the job demanded skills as: 1) an analytical chemist for determining by-products and potential side reactions, 2) a chemical engineer for scaling the process up for full scale production, 3) a background in health and safety to examine effects of reactants and products to workers and 4) a working knowledge of cost evaluation to determine finances, manpower requirements and time required for large scale production of chemicals.

1983-1995 Officer United States Air Force

May 1985 to April 1987 ***Program Manager for Joint STARS radar system.*** Evaluated proposals from three major contractors for technical risk and cost trade-offs for Joint STARS radar system. Created, reviewed and evaluated technical specifications and developed and coordinated cost, schedule, and technical management plans necessary for Milestone 1 decision. Managed the Pre-planned Product Improvement (P3I) Block II effort to develop future capabilities of the Joint STARS system.

May 1987 to Nov. 1990 ***Solid State Research Scientist and Program Manager for Rome Air Development Center.*** Duties included design and evaluation of experiments in material sciences, management of research contracts and supervision of government civilian and contractor personnel.

May 1987 to Sept. 1988 ***Research Scientist in High Temperature Superconductivity (HTSC).*** Studied theory and explored alternative materials for HTSC.

Oct. 1988 to Nov. 1990 ***Program Director for Bulk Growth Indium Phosphide Research Initiative.*** As team leader of this project, I led three

government civilians and 1 contractor in developing a new process for growth of indium phosphide. As a result of this work several papers and patents were published and the project was highlighted by the Air Force Office of Scientific Research (AFOSR) in its briefing to the Department of Defense.

Feb 1990 to Nov 1990 ***Acting Deputy Director for Electronic Sciences Directorate of Rome Laboratories (RADC/ES).*** Responsible for day to day operations of RADC/ES to include meeting and briefing visiting dignitaries, preparation of Technical Area Plans (TAPS) for RADC, and Defense Management Review initiatives to consolidate laboratory functions.

Nov. 1990 to July 1992: **Project Officer for Space Missions Advanced Plans and Program Manager for Military Satellite Communications in Electronic System Division (ESD/XR) Space Missions Directorate**. Served as the Air Force focal point in a major DOD Military Satellite Communication architecture study initiated by Mr Andrews, the Assistant Secretary for Defense. Also, represented ESD during the Air Staff MilSatCom Summit in support of Gen. McPeak, Chief of Staff of the Air Force. Other prime responsibilities include review of all Air Force Space Command operational requirement documents for technology shortfalls, translate these requirements into system architecture road maps and guide Rome Laboratory in developing its product technology plans.

AWARDS

Commendation Medal for work on Joint STARS System
Commendation Medal for Research efforts at Rome Laboratory
Commendation Medal for Space Communication architecture in Advanced Plans and Program office
Achievement Medal for breakthrough in Indium Phosphide Crystal Growth
Air Force Organizational Excellence Ribbon presented to Electronic System Division
Air Force Organizational Excellence Ribbon presented to Rome Laboratory
Air Force Organizational Excellence Ribbon presented to Electronic System Division
Scientific Achievement Award for Research on Investigation of Possible superconductivity in Titanium Boride. 11JUN91
Scientific Achievement Award for Research on MLEK Crystal Growth of <100> Indium Phosphide
Travel Award for Poster presentation at Ohio Valley Society of Toxicology meeting September 1994
Carcinogenesis Specialty Section Award at National Society of Toxicology meeting for Research on Oxidative Stress March 1995

EDUCATION

Civilian

Bachelor of Science in Chemistry from Wagner College 1978 (GPA 3.95)

Bachelor of Science in Electrical Engineering from Ohio University 1985 (GPA 3.63)

Master of Science in Electrical Engineering: solid state devices concentration 1989 (GPA 3.85) University of Massachusetts at Lowell

Ph.D. in Toxicology at Indiana University School of Medicine August 1995

Military

Squadron Officer School
Certified Level I Acquisition Manager
Certified Level I Developmental Engineering
Certified Level I Scientist Professional Development
Certified Level I Scientific Manager Professional Development

Management Courses

Introduction to Acquisition Management Aug. 1985
DMSC Technical Management Course March 1986
DMSC Test and Evaluation Management Course April 1989
AFSC Management Course (Sys 200) July 1990

Technical Specialty Courses

Modern Material Analysis Techniques (Material Research Society)
Opto-Electronic Materials, Processes, and Devices (Material Research Society)
Vapor Phase Epitaxy (Material Research Society)
Radar: Past, Present and Future IEEE
Microwave High-Power Tubes and Transmitters

PUBLICAITONS

"Magnetically Stabilized Kyropoulos Growth of Undoped InP", Material Letters, **8(11,12):** 486-488 (1990)

"MLEK Crystal Growth of (100) Indium Phosphide", Journal of Electronic Materials, **20(12):** 967-971 (1991)

"Magnetically Stabilized Kyropoulos and Czochralski Growth of InP" Proceedings of the Second Annual Conference on Indium Phosphide and related materials (1990)

"MLEK crystal Growth of (100) Indium Phosphide", Proceedings of the Third Annual Conference on Indium Phosphide and related materials (1991)

Investigation into the possible superconductivity of Titanium Diboride", Fall Proceedings Material Research Society (1989).

Bachowski, S., Baker, T., Stevenson, D., Walborg, E., and Klaunig, J.E. (1995): The potential role of oxidative stress in nongenotoxic carcinogenesis in the mouse liver. In Slaga, Goldsworthy, Henry and Stevenson (Eds), In Progress in Clinical and Biological Research: Growth Factors and Tumor Promotion: Implications for Risk Assessment Conference, Barton Creek, TX. , Wily and Liss, NY. pp385-396.

Baker, T., Bachowski, S., Stevenson, D., Walborg, E., and Klaunig, J.E. (1995): Modulation of gap junctional intercellular communication in rodent, monkey and human hepatocyte by nongenotoxic compounds. In Slaga, Goldsworthy, Henry and Stevenson (Eds), In Progress in Clinical and Biological Research: Growth Factors and Tumor Promotion: Implications for Risk Assessment Conference, Barton Creek, TX. , Wily and Liss, NY. pp71-80.

Bachowski, S. and Klaunig, J.E. (submitted 1995): Reactive Oxygen Species Generation in Liver Microsomes and Hepatocytes by the Organochlorine Compound Dieldrin.

Bachowski, S., Kolaja, K.L., Xu, Y., Ketcham, C. A., Stevenson, D.E., Walborg, E.F., and Klaunig, J.E. (submitted 1995): Role of Oxidative stress in Dieldrin Hepatocarcinogenesis in the B_6C_3F1 Mouse

Bachowski, S., Xu, Y., Stevenson, D.E., Walborg, E.F., and Klaunig, J.E. (submitted 1995): Dieldrin Induction of Oxidative Stress in Rodents

Bachowski, S., Ketcham, C.A., Stevenson, D.E., Walborg, E.F., and Klaunig, J.E. (submitted 1995): Modulation of Dieldrins induction of Oxidative Stress in the in the B_6C_3F1 Mouse by Dietary Vitamin E

Bachowski, S., Xu, Y., Baker, T., Stevenson, D.E., Walborg, E.F., and Klaunig, J.E. (draft): In vitro Analysis of dieldrin in the B_6C_3F1 mouse and F344 rat: Implications of Oxidative Stress.

Abstract

Baker, T., Bachowski, S., Stevenson, D., Walborg, E., and Klaunig, J.E. (1993): Modulation of Gap Junctional Intercellular Communication in Rodent, Monkey and Human hepatocytes by Organochlorine Pesticides. Growth Factors and Tumor Promotion: Implications for Risk Assessment Conference, Barton Creek, TX.

Xu, Y., Bachowski, S., Stevenson, DE, Walborg, EF Jr., Klaunig, JE., (1994): A relationship between oxidative stress and species susceptibility to hepatocarcinogenesis induced by dieldrin and phenobarbital in B_6C_3F1 mice and F344 rats. Thirteenth summer Symposium in Molecular Biology: Molecular and Cellular Mechanisms of Toxicity August 3-5, 1994 Penn State University, University Park, PA.

Bachowski, S., Xu,Y., Stevenson, DE, Walborg, EF Jr., Klaunig, JE., (1995): Role of oxidative stress in dieldrin induced mouse hepatocarcinogenesis. The Toxicologist. 15(1): 200.

Xu, Y., Bachowski, S., Stevenson, DE, Walborg, EF Jr., Klaunig, JE., (1995). Correlation between the induction of oxidative stress and rodent susceptibility to hepatocarcinogenesis. The Toxicologist. 15(1): 200.

Ketcham, CA, Bachowski, S., Stevenson, DE, Walborg, EF Jr., Klaunig, JE., (1995): Effect of vitamin E on dieldrin induced hepatic DNA synthesis and neoplasia in mice. The Toxicologist. 15(1): 216.

Klaunig, JE., Xu, Y., Bachowski, S., Ketcham, CA, Isenberg, KL, Baker, TK, Walborg, EF Jr., stevenson, DE, (1995): Oxidative Stress in nongenotoxic carcinogenesis. International Congress of Toxicology-VII July 2-6, 1995

Nie, Y., Bachowski, S., Knoblach, S.M., Schoepp, D.D., Klaunig, J.E., and Kubek, M.J. (1995): Potassium Induced Changes in Thyrotropin-Releasing Hormone (TRH), Glutamate, and Lactate Dehydrogenase Release from Hippocampal Slices after Seizure. The Endocrine Society. Washington DC convention center June 1995.

Nie, Y., Bachowski, S., Klaunig, J.E., and Kubek, M.J. (1995): Superfusion of Hippocampal Slices: evaluation of Neuronal Degeneration by LDH Assay. Society For Neuroscience.

PATENTS

Patent #5,376,626 entitled "Magnetic Field Operated superconductor Switch" 14MAR95

Patent #5,431,125 entitled "Twin-Free Crystal Growth of III-V Semiconductor Material" 11JUN95

Air Force Invention #19824 entitled "Twin-Free Crystal Growth of III-V Semiconductor Material" 16JUL92

Air Force Invention #18,289 entitled "Magnetic Field Operated Superconductor Switch and Method for Switching" 19MAR91

Air Force Invention #18,239 entitled "Non-obscuring Viewing Port for crystal growth apparatus" 19MAR91

Air Force Invention #19,476 entitled "Capillary Pressure Relief for Magnetic Kyropoulos Growth of Semiconductor Crystals" 19MAR91

Pending "Flat crown (100) Magnetically stabilized growth of Indium Phosphide and related materials"

Pending "Kinetically controlled in-situ synthesis of indium phosphide via phosphorus injection"